Microbial Diversity and Community Changes in High Mountain Habitats

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Summary

Attempts to discover novel microorganisms possessing specific metabolic properties have led to an intensified search and exploration of extreme environments. Polar regions, deep oceans, high mountain environments with their glaciers, snow covers and cold water lakes belong to the habitats where psychrotolerant and psychrophilic ("cold loving") microorganisms exist and where cold-active enzymes are needed.

Although mountain environments have been exploited for a number of purposes, the giant microbial gene pool present in the extreme habitats has been neglected so far. Our studies reveal a large microbial diversity, including cold-adapted bacteria and archaea which are present in high mountain lakes, snow fields and glacial ice habitats. The microbial diversity of both, autochthonous (endemic) and allochthonous microorganisms play essential ecological roles in food web maintenance under the low-nutrient conditions in these habitats.

The present study was carried out at the Jöri and Tambo lakes, nearby snowfields and glaciers, and at higher altitude snow packs and glacial ice at Jungfraujoch. The Jöri and Tambo sites are situated in the eastern Swiss Alps, at altitudes between 2'400 m and little above 3'000 m, whereas the Jungfraujoch site (JFJ) is located in the central Swiss Alps, at an altitude of approximately 3'500 m. The conditions in all these high altitude ecosystems are characterized as extreme with a long period of ice and snow-cover, low water temperatures ranging in lakes from 0 to 12ºC, and typically low-nutrient concentrations.

Culture-dependent and molecular techniques (SSU rRNA gene-based analyses) were used to assess the diversity of the microbial communities from these habitats. Specific, sensitive, and robust methods such as the Polymerase Chain Reaction (PCR), Restriction Fragment-Length Polymorphism (RFLP), cloning, Fluorescent *In-situ* Hybridization (FISH), and sequencing were used for the detection and molecular characterization of a large number of extremophiles. Seasonal microbial community structures and dynamics over temperature variations were studied using Temporal Temperature Gradient Gel Electrophoresis (TTGE).

Phylogenetic analysis of the culturable bacteria originating from the Jöri and Tambo catchments showed that they fall into 5 major phylogenetic groups (Chapter 2). They affiliate with the *Proteobacteria*, consisting of the *alpha-, beta-,* and *gamma-* subgroups (84.4%), the *Gram-positive high G+C* group (13.1%), and the *Cytophaga-Flavobacterium-Bacteroides* group (2.4%). The results from growth experiments at various temperatures indicate that bacteria occupying these extreme habitats are psychrotolerant or mesophilic bacteria exhibiting a broad temperature tolerance and possess the capability to adapt to life at low-temperatures.

The planktonic microbial communities were studied in Jöri Lake XIII (2'640 m) (Chapter 3). Spatial and temporal changes in the composition of the microbial communities occupying this lake habitat were demonstrated. TTGE analysis yielded interesting seasonal banding patterns. While the lake was stratified it showed distinct banding patterns, whereas the patterns were identical at all depths during summer upwelling events. The TTGE patterns obtained from environmental community DNA were well represented by the 16S rDNA fragments present in the clone library.

Our results support the selective adaptation model; there the microbial community experiences high environmental fluctuation, adapting itself by continuously selecting for those members which are best suited to the particular conditions. Out of 45 TTGE bands, 32 were unique whereas 6 out of the remaining 13 were predominant strains present at more than one sampling period. Sequence analysis of 38 TTGE bands showed 88% to 99% similarity to 16S rRNA gene sequences present in databanks. Phylogenetic analysis revealed that the predominant populations belong to the *alpha-, beta-,* and *delta-* subgroups of the *Proteobacteria*, the *Actinobacteria*, the *Verrucomicrobia*, the *Planctomycetes*, and to the chloroplasts of a unicellular green alga. FISH and the sequencing analyses showed that the highest bacterial diversity stems from the group of the *beta-Proteobacteria*, which also showed the highest seasonal dynamic in Jöri Lake XIII. The archaeal clone libraries were phylogenetically affiliated with both Crenarchaeota and Euryarchaeota lineages originating from temperate and hyperthermophilic methane-rich habitats. The *in vitro* experiment showed that communities shift dynamically in response to the variable environmental conditions, such as nutrient availability, light intensity and the presence or absence of oxygen (Chapter 5).

The diversity of the snow cover communities from Jöri and Jungfraujoch (JFJ) habitats was quite different. The most diverse snow communities were present in the colored snow from sites at lower altitudes, which contain algal blooms (Chapter 4). The snow habitats revealed communities which consisted of prokaryotic and eukaryotic phyla. The majority of Jöri snow clones belong to the *beta-Proteobacteria* subgroup, whereas the *gamma-Proteobacteria* represented only a minor portion of the total clone library. The non-proteobacterial clones corresponded to the *Verrucomicrobia,* the *Actinobacteria,* the *Bacteroidetes,* and to the *chloroplasts* and *mitochondria* of eukaryotic organisms. The eukaryotic domain was represented by *Chlorophyta, Spermatophyta, Fungi,* and *Cercozoa*. In the JFJ snow habitat, the *Actinobacteria* were dominant whereas the *beta-Proteobacteria* subgroup was the most abundant in the JFJ ice habitat. Some JFJ snow clones corresponded to bacteria of human-origin and to Crenarchaeota, which were not present in snow at lower altitudes.

These results show a broad microbial diversity and dynamic population changes in high altitude aquatic habitats with harsh and highly fluctuating environments. For bioprospecting they represent an untouched reservoir of genomes which might be exploited for biotechnological purposes, e.g. for cold active enzymes.

Zusammenfassung

Das Interesse an “neuen” Mikroorganismen mit speziellen Stoffwechseleigenschaften führte zu einer intensivierten Suche und Erforschung von extremen Lebensräumen. Die Polarregionen, die Tiefsee, die Hochgebirge mit ihren Gletschern, Schneedecken und Kaltwasserseen gehören zu den Habitaten, in denen psychrotolerante und psychrophile (“Kälte-liebende”) Mikroorganismen existieren und wo mit grosser Wahrscheinlichkeit an die Kälte angepasste Enzyme gefunden werden können.

Obwohl das Gebirge schon für viele Zwecke ausgebeutet wurde, hat man bis heute den gigantischen mikrobiellen Genpool in diesen Extremhabitaten nicht wirklich erforscht. Unsere Studien enthüllen eine grosse mikrobielle Diversität. Autochthone (endemische) und allochthone Mikroorganismen spielen eine wichtige ökologische Rolle in der Aufrechterhaltung der Nahrungsnetze unter den meist oligotrophen Bedingungen.

Die vorliegende Arbeit wurde an den Jöri- und Tambo-Seen ausgeführt. Die Habitate sind umgeben von Schneefeldern und Gletschern und, in noch höheren Lagen (auf dem Jungfraujoch), von ewigem Schnee und Gletschereis. Die Jöri und Tambo Regionen liegen im östlichen Teil der Schweizer Alpen in Höhen zwischen 2400 m bis knapp über 3000 m ü. M., wohingegen die Jungfraujochregion (JFJ) in den Zentralalpen auf einer Höhe von ungefähr 3500 m ü. M. liegt. Die generellen Lebensbedingungen in den Hochgebirgsseen Jöri und Tambo werden, wegen ihrer langen Schnee- und Eisbedeckungsperioden, ihren tiefen Wassertemperaturen zwischen 0ºC und maximal 12ºC, und ihren typischerweise tiefen Nährstoffkonzentrationen, als extrem bezeichnet.

Kulturabhängige und molekularbiologische (ssu rRNA Gen-Analyse) Methoden wurden angewandt, um die Diversität der mikrobiellen Gemeinschaften in diesen Habitaten zu erfassen. Spezifische, sensitive und robuste Methoden, wie die Polymerasekettenreaktion (PCR), Restriktionsfragmentlängenpolymorphismus-Analyse (RFLP), Klonierung, Fluoreszenz-In-Situ-Hybridisierung (FISH) und Sequenzierung wurden für die Detektion und die molekulare Charakterisierung einer grösseren Anzahl von Extremophilen eingesetzt. Die Veränderung der mikrobiellen Gemeinschaftsstruktur über die Jahreszeiten, sowie deren Wechsel während Temperaturschwankungen wurden mittels “Temporaler Temperatur Gradienten Gelelektrophorese” (TTGE) aufgezeichnet.

Die kultivierten Bakterien, die aus der Jöri und der Tambo Gegend stammen, können fünf grösseren phylogenetischen Gruppen zugeordnet werden (Kapitel 2). Sie fallen in die *alpha-, beta-, gamma-* Gruppen der *Proteobakterien* (84.4%), in die Gruppe der *Gram-positiven mit hohem G+C Gehalt* (13.1%), und in die *Cytophaga-Flavobacterium-Bacteroidetes*-Gruppe (2.4%). Die Resultate der Wachstumsexperimente bei verschiedenen Temperaturen zeigen, dass die Bakterien, die diese

Extrem-Habitate bewohnen, psychrotolerante oder mesophile Bakterien mit einem breiten Temperaturtoleranzspektrum sind. Sie sind fähig, sich an ein Leben bei tiefen Temperaturen anzupassen. Wie die Wachstumsraten zeigen, gibt es Bakterien, die bei tiefen Temperaturen ein breites Spektrum an Nährstoffkonzentrationen akzeptieren. Andere wiederum wachsen nur bei höheren Temperaturen und verschiedenen Nährstoffkonzentrationen gut.

In Jörisee XIII (2640 m ü. M.) wurden die mikrobiellen Planktongemeinschaften untersucht (Kapitel 3). Es wurden räumliche und zeitliche Wechsel in der Zusammensetzung der mikrobiellen Gemeinschaften in diesem Habitat gefunden. Die TTGE Analyse lieferte interessante saisonal verschiedene Bandenmuster. Während der geschichteten Phase des Sees ergaben sich unterschiedliche Bandenmuster für verschiedene Tiefen, wohingegen die Muster bei Durchmischungsereignissen während des Sommers in allen Tiefen identisch waren. Die DNA TTGE-Muster der gesamten mikrobiellen Gemeinschaft im See waren gut vertreten in den Klonbibliotheken von 16S-rDNA Fragmenten. Die von extrahierter und amplifizierter DNA aus Umweltproben erhaltenen TTGE Muster stimmen gut überein mit den 16S-rDNA Fragmenten aus der angelegten Klonbibliothek, welche die in TTGE Banden auftretenden, vorherrschenden Bakterien repräsentieren.

Unsere Resultate unterstützen das „Selektive Anpassungsmodell“, in welchem die mikrobielle Gemeinschaft in Ökosystemenen mit hohen Schwankungen in den Umweltbedingungen sich selbst kontinuierlich anpasst, indem sie diejenigen Organismen selektioniert, die am besten an die speziellen Bedingungen adaptiert sind. Von 45 TTGE-Banden kamen 32 nur einmal vor, wohingegen von den 13 verbleibenden 6 zu vorherrschenden Stammlinien gehörten, die in mehr als einer Probenahmeperiode vorkamen. Die Sequenzanalyse von 38 TTGE Banden zeigte 88% bis 99% Übereinstimmung mit 16S rRNA Gensequenzen in der GenBank. Die phylogenetische Analyse deckte auf, dass die dominanten Populationen zu den *alpha-, beta-,* und *delta-* Untergruppen der *Proteobakterien*, sowie zu den *Actinobakterien*, den *Verrucomikrobiern*, den *Planktomyceten* und *Chloroplasten* von einzelligen Grünalgen gehören. FISH und Sequenzanalysen zeigten weiter auf, dass die höchste bakterielle Diversität aus der Gruppe der *beta-Proteobakterien* stammte, welche auch die grösste saisonale Dynamik im Jörisee XIII aufwies. Die Klone der Archäen-Klonbibliothek fielen phylogenetisch sowohl in die Gruppe der Crenarchaeota, als auch in die der Euryarchaeota, wobei sie sich in die Nähe von Archäen eingliederten, die aus temperierten und sogar hyperthermophilen, methanreichen Habitaten stammen. Das *in vitro* Experiment zeigte, dass mikrobielle Gemeinschaften sich dynamisch verschieben als Antwort auf sich verändernde Umweltbedingungen, wie Nährstoffverfügbarkeit, Lichtintensität und die Präsenz oder Abwesenheit von Sauerstoff (Kapitel 5).

Die Diversität der Schneedecken-Gemeinschaften von den Jöri- und JFJ-Habitaten war stark verschieden. Die am stärksten diverse Schneegemeinschaft fand sich im gefärbten Schnee von tieferen Lagen (Jöri), die Algenblüten enthielten (Kapitel 4). Die Schneehabitate enthielten Gemeinschaften, welche aus prokaryotischen und eukaryotischen Stämmen bestanden. Die Mehrheit der Jöri-Schnee

Klone gehört zu den *beta-Proteobakterien*; die *gamma-Proteobakterien* waren nur in einem kleineren Teil der ganzen Klonbibliothek vertreten. Die nicht-proteobakterialen Klone liessen sich in die *Verrucomikrobier,* die *Aktinobakterien,* die *Bakteroideten*, sowie *Chloroplasten* und *Mitochondrien* von eukaryotischen Organismen einteilen. Die eukaryotische Domäne wurde durch *Chlorophyten*, *Spermatophyten*, *Pilze* und *Cercozoen* repräsentiert. Im JFJ-Schnee waren mehrheitlich *Aktinobakterien* zu finden, wohingegen im JFJ-Eis die *beta-Proteobakterien* die häufigsten waren. Einzelne JFJ Schneeklone stammten von Bakterien menschlichen Ursprungs und aus der Gruppe der Crenarchäen, welche nicht vorhanden waren in Schnee von tiefen Lagen.

Diese Resultate geben Einblick in eine breite mikrobille Diversität und zeigen einen dynamischen Populationswechsel in hochalpinen Gewässer- und Schneehabitaten mit harschen, stark veränderlichen Umweltbedingungen auf. Hochalpine Habitate repräsentieren fast unberührte Genomreservoire, welche noch nicht weiter für biotechnische Zwecke, wie z.B. in der Kälte aktive Enzyme, erforscht wurden.

Introduction

High mountain lake habitats as indicators of environmental changes

In recent years, interest in the presence and survival of microorganisms which inhabit cold and nutrient limited ecosystems has been increasing. Life strategies which evolved in the prokaryotic world have attracted the attention of microbiologists for many years. Currently, microbial life at low temperatures has also attracted the interest of those who are studying conditions for life on other planets, such as Mars and Europa. Naturally cold ecosystems, such as Antarctic and Arctic regions, the deep sea, high altitude mountain environments and glaciers may represent analogs on earth to the cold extraterrestrial habitats.

High mountain ecosystems are also of ecological interest because of their richness in natural resources, their biodiversity, the clean water, healthy climate, original soil, and diverse geology, which are relevant to issues of environmental protection. On the other hand, the utilization of the mountain areas e.g. by the tourist industry is an issue of socio-economic importance.

For many decades mountain areas have been used as sites to evaluate possible effects of global warming and its consequences. They are sensitive indicators for the early detection of the impacts of rapid climatic changes on the hydrological cycle, water chemistry and ice melting. Even a small temperature change can strongly influence their hydrological cycle (Schindler et al. 1990). The climatic warming observed in the last 25-30 year seems to be most pronounced in the mountain regions all over the world (Beniston et al. 1997, Rogora et al. 2003). The temperatures measured in these areas have increased by 1.5 – 2.0ºC on the average since 1980, whereas an increase of approximately 0.5ºC was observed on a global scale (Beniston et al. 1997). The climatic changes and environmental fluctuations particularly affect open waters of high mountain lakes (Psenner and Schmidt 1992; Sommaruga-Wögrath et al. 1997; Koinig et al. 1998). Besides climatic changes which significantly influence the hydrochemistry of the lakes in the high altitude areas, other factors, such as the atmospheric dust deposition (Psenner 1999) and long periods of anthropogenic impacts (Kamenik et al. 2000) also shape the ecology of these areas.

The duration and extent of the snow cover in a lake catchment are dominant factors governing the release of weathering products from rocks and soils into the water (Wright and Schindler 1995). Recent study showed that the effect of climate warming has been influencing the weathering rate in the mountains (Rogora et al. 2003). Through the chemical and physical degradation of rocks and soil minerals, solute contents were increasing. Warmer temperatures are also enhancing biological processes by increasing the primary productivity.

There are a number of other factors that might influence long term changes in the chemical characteristics of alpine lakes (Kamenik et al. 2000). Airborne desert dust depositions contain major basic cations, carbonates, sulphates and other anions of nutrient value for remote lakes (Psenner 1999). The high calcium contents of the Saharan aerosols, which are also found in aerosols from other desert, might significantly contribute to the biogeochemical cycles involving acid neutralization (De Angelis

and Gaudichet 1991). Dust depositions might be one of the key factors for the development of high mountain lakes. Airborne dust in snow and rain might contribute to the high buffering capacity of some mountain lakes and to the elevated seasonal nutrient levels (De Angelis and Gaudichet 1991; Psenner 1999).

Earlier studies carried out in mountain lake habitats were based on chlorophyll *a* measurements and biochemical productivity analyses. They showed active microalgal development and distinct microbial communities during the short ice-free period (Hinder et al. 1999a; Hinder et al. 1999b). These authors found that the timing of lake ice melting influences the seasonal succession of the phytoplankton community. Despite low nutrient input and commonly limited concentrations, some lakes showed self-trophication capabilities, which is particularly obvious during the summer season (Iqbal-Nava 2003). Microorganisms living in phosphate-limited mountain lakes were able to overcome this limitation by mobilizing particulate iron phosphates from sedimentary deposits and biofilm-associated microbes can develop on iron-phosphate-oxyhydroxide coated surfaces (Amberg-Brunner 2002).

The role and diversity of microbial communities in high altitude habitats

Microorganisms dwelling in extreme environments have attracted microbial ecologists because of the uniqueness of peculiar physiological properties. The Antarctic, Arctic, as well as high altitudes contain habitats which are representative for cold environments. These habitats often provide good models for ecosystems with a simple and short food web and for studies of microbial dynamics at cold temperatures. Organisms that live and actively grow at near freezing temperatures face a number of growth-limiting constraints. Under the cold conditions the enzyme reaction rates are generally lower, uptake and transport systems function more slowly, membranes become less fluid and nucleic acid structures become more stable (Feller et al. 1996; Graumann and Marahiel 1996). Microorganisms have evolved various ways to adapt (Gerday et al. 1997); for instance, cold active proteins have evolved which are catalytically efficient at temperatures as low as 0ºC (Gerday et al. 1997).

Microorganisms living at high altitudes are also challenged by intense UV radiation. UV B radiation in high mountain winter covers, can reach up of 50% higher values than at sea level (Psenner and Sattler. 1998). The strong UV exposure can lead to growth inhibition of benthic diatoms (Bothwell et al. 1994); causes damage in heterotrophic flagellates (Sommaruga et al. 1996), and inhibits the rate of nutrient uptake by bacterioplankton in the water column (Sommaruga et al. 1997). Attenuation coefficient (K_d) values, which are based on the upper and lower UV radiation intensities, are used to illustrate the strength of UV intensity at different wave lengths i.e. 305, 320, 340, 380 nm. Results from Iqbal-Nava's study (2003) showed that the diffuse attenuation coefficients (K_d) measured in Lake Jöri XIII were higher (ranging from 0.68 to 1.54) than those in other high mountain lakes. K_d values of the Austrian Lake Gossenkölle at 2'417 m a.s.l. ranged from 0.14 to 0.32 (Sommaruga and

Psenner 1997), K_d values of the Argentinean Lago Schmoll in 2'000 m a.s.l. were between 0.07 and 0.17 (Morris et al.1995), whereas the mean K_d values of 13 oligotrophic lakes in the Bariloche region in Argentina were between 0.3 and 0.8 (Morris et al 1995).

High altitude lakes are normally situated above the timberline and have catchment areas with little or no vegetation. Consequently, they are often oligotrophic as defined by the concentration of nitrogen and phosphorus. Examples are the MOLAR lakes such as in the High Tatra Mountains (Slovakia, 2'655 m a.s.l.) (Kopáček et al. 1995), and the Sumava Mountains (Czech Republic, 1'456 m. a.s.l.) (Kopáček et al. 1995); Schwarzsee (Austria, 2'799 m a.s.l.), Lago Paione Superiore (Italy, 2'269 m a.s.l.) (Mosello et al. 2002), Yellow Belly Lake (U.S.A, 2'157 m a.s.l.) (Pilati and Wurtsbaugh 2003), Gossenköllesee (Austria, 2'417 m a.s.l.) (Kamenik et al. 2000), and the Jöri lakes III and VII (Switzerland, 2'557 m a.s.l.) (Hinder et al. 1999a). Lake Jöri XIII does not meet the criteria for oligotrophy especially in summer season. Low N:P ratios were measured at the end of the ice covered period, whereas the highest ratios were measured during the ice free period (Iqbal-Nava 2003). The physico-chemical conditions strongly depend on the duration of the ice and snow cover. The nutrient conditions in Lake Jöri XIII initiate high biomass and remarkable microbial activities and population dynamics.

In aquatic ecosystems, the bacterioplankton community is an integral part of the food web, which is essential for protists and metazoans (Cho and Azam 1988). Little information is available, however, on the temporal and spatial alterations of the microbial community in pelagic food webs of high mountain lake habitats (Felip et al. 1995). Recent studies focused on more specific subjects such as the effect of the UV radiation on bacteriovory (Sommaruga et al. 1996), microbial diversity and activity (Alfreider et al. 1996) and palaeolimnology (Koinig et al. 1998). Compared to eutrophic ecosystems, the pelagic food webs in these oligotrophic ecosystems appeared to be less complex and the microbial loops might play an essential role in recycling nutrients for the higher trophic levels (Hinder et al. 1999a and 1999b). The significance of the pelagic microbial assemblage increases with the level of oligotrophy of the lake water (Hahn et al. 1999). This is true for pelagic food webs in circumcontrol as well as in acidified high mountain lakes (Wille et al. 1999).

Molecular approaches applied to the study of high mountain aquatic ecosystems

Before the availability and the widespread application of molecular genetic techniques in microbial ecology, the microbial diversity was underestimated since many studies were mainly based on microscopic observations and on the culturable microorganisms. It is now known that only a few percent of the microorganisms are actually cultivable (Amann et al. 1995). The high discrepancy between total cell counts using epifluorescence microscopy and the number of colonies obtained by the different cultivation effort is due to several reasons, e.g. the lack of knowledge of the growth supporting microenvironmental conditions and the interpendency between different microorganisms.

Currently, 26 phyla of the approximately 52 identifiable major phyla within the bacterial domain have cultivated representatives (Rappé and Giovannoni 2003). The polymerase chain reaction (PCR), invented in 1983 by Karry Mullis, has enabled us to also assess the diversity of uncultivated microorganisms. Nucleic acids extracted from environmental samples are suitable materials to describe the diversity through nucleotide sequence comparisons, without the need to culture organisms.

When Carl Woese introduced the use of 16S rRNA sequences for molecular phylogeny (Woese 1987), only 12 microbial phyla could be phylogenetically compared. Nowadays more than 30,000 environmentally retrieved bacterial 16S rRNA genes are available (Rappé and Giovannoni 2003; Wagner 2004). Carl Woese's approach has also uncovered a new domain of life, the Archaea, a distinct microbial world that in earlier time was recognized as the Archaebacteria. Formerly, Archaea were thought to exclusively consist of thermophiles, halophiles, and strictly anaerobic methanogenic microorganisms inhabiting extreme environments. Only recently has the knowledge about the Archaea been raised enormously. Today, Archaea are being recognized as ubiquitous microorganisms, also present in low temperature marine habitats, in which they were found in high abundances (DeLong et al. 1994). However, little information is available at present about the Archaea, their identity and abundances in low temperature, high altitude habitats.

In recent years, advanced techniques have been developed to study microbial communities. PCR-based community fingerprinting techniques such as Temporal Temperature Gradient Gel Electrophoresis (TTGE) or Denaturing Gradient Gel Electrophoresis (DGGE) allow us to distinguish the microbial composition based on community banding patterns. These techniques are based on the separation of the PCR products of genes isolated from mixed populations possessing different nucleotide sequences. By applying these techniques, the diversity and the community structure can be described (Muyzer et al. 1993; Muyzer and Smalla 1998; Muyzer 1999). In many different environments, the population dynamics and the community succession can be followed seasonally as well as spatially (Øvereas et al. 1997; Crump et al. 2003; Brümmer et al. 2003).

A direct and rapid detection technique to taxonomically identify the member of microbial communities is Fluorescent *In-Situ* Hybridization (FISH). The use of specific rRNA-targeted oligonucleotide probes allows one to determine species, special groups, or domains quantitatively without the need to culture them (Amann et al. 1990; Amann et al. 1995). The *in-situ* assessment for the abundances and the microbial composition has been successfully applied to various habitats. Different studies were carried out to investigate the community compositions of habitats either from freshwater high mountain lakes (Alfreider et al. 1996; Pernthaler et al. 1998; Tonolla et al. 1999; Glöckner et al. 1999; Gattuso et al. 2002) or from marine habitats (Glöckner et al. 1999; Cottrell and Kirchman 2000; Dang and Lovell 2002; Pernthaler et al. 2002; Pernthaler et al. 2003).

The PCR-based methods (culture-independent analyses) also have their limitations. Without having appropriate culture representatives, it is quite difficult to predict phenotypic properties of the

detected but uncultured microorganisms. It is a challenge for microbial ecologists to assign functions and activities of populations within those communities in complex ecosystems (Wagner 2004; Paerl and Steppe 2003). The microbial communities, which consist of mixed groups of microbial species having different functions and metabolic activities, are responsible for maintaining ecosystem fitness (Paerl and Steppe 2003), i.e. microbially-mediated chemical transformations and habitat alterations (Boetius et al. 2000).

Research questions

This study is based on the following questions:

- What is the microbial diversity present in high mountain lake habitats? Are there microorganisms which are new and characteristic for the extreme conditions?
- Are psychrotolerant organisms phylogenetically closely related to each other?
- Do microbes present in oligotrophic cryo-habitats possess special life strategies?
- Do the communities possess physiological and ecological flexibilities with which they can respond to the fluctuating physico-chemical conditions?
- What are the ecological parameters determining the community structures and how rapidly do they respond to environmental changes?

Objectives of this study

The aim of the present study was to assess the microbial diversity in high mountain lakes and snow cover habitats and to follow the dynamic changes of the microbial population in response to the fluctuating environmental conditions. The study is based on a combination of culture-dependent methods and PCR-based molecular approaches. Molecular techniques provide excellent and powerful tools for the study of the microbial populations living in these extreme habitats. Several studies have been carried out in these habitats, however, little is known about the microbial diversity, the physiological strategies and the flexibility of the communities to adapt to the various environmental fluctuations.

According to the particular topic in which the questions were addressed, this thesis is subdivided into three main parts: The molecular identification and phylogenetic affiliation of heterotrophic bacteria from high mountain lake habitats (Chapter 2); the microbial diversity and population shifts in response to environmental fluctuations in Lake Jöri XIII (Chapter 3); and the diversity of the bacteria in snow cover habitats (Chapter 4). Chapter 5 summarizes preliminary result from an *in vitro* community shift experiment, which is mainly related to Chapter 3.

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Phylogenetic affiliation and growth characteristics of heterotrophic bacteria from nutrient-limited, cold high mountain lake habitats

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Abstract:

We investigated the cultivable heterotrophic bacteria from cold, oligotrophic high mountain lake habitats. Pure cultures were isolated by direct plating using low nutrient medium and growth at temperatures between 3 and 4°C. Characterization was performed employing phenotypic and molecular methods. Several isolates were capable of expressing an ice nucleation phenotype. Universal primers for Bacteria (S-D-Bact-0008-b-S-20 and S-D-Bact-1524-a-A-18) were used for PCR to amplify almost the full length of the 16S rRNA genes of 84 selected isolates. Restriction fragment length polymorphism (RFLP) analysis with *Hinf*I and *Hae*III was used to identify 15 different patterns. The different isolates were further analysed by TTGE (Temporal Temperature-gradient Gel Electrophoresis) and sequenced. Based on the phylogenetic analysis, the isolated bacteria belong to 5 major phylogenetic groups: the *alpha-*, *beta-*, and *gamma-Proteobacteria* (84.4%), the *Actinobacteria (Gram positive High G+C bacteria)* (13.1%), and the *Bacteroidetes* (*Flavobacterium-Cytophaga-Bacteroides* group) (2.4%). Isolate J07 which showed maximal 96.7% sequence similarity to the closest relative in the databank represents a candidate for a novel species of the genera *Clavibacter* sp. Little is known about the adaptation of these extremophiles to rapid changes of environmental conditions. Our results demonstrate that bacteria isolated possess the flexibility of growing at various temperatures and nutrient concentrations, suggesting that they are psychrotolerant or mesophilic. The bacterial community which is composed of a number of ecotypes with various heterotrophic traits possesses the capability to adapt to changing temperatures and to low nutrient concentrations.

1. Introduction

Remote, high altitude lakes of the Alps are extreme habitats, which experience high environmental fluctuations and are not heavily influenced by anthropogenic activities. Aquatic habitats in slowly deglaciating areas evolve under extreme physical conditions and commonly low nutrient concentrations (oligotrophic). Seasonal and diurnal temperature variations, sudden weather fluctuations and hydrological changes during the ice-free period, as well as long ice-covered periods over the year, provide an environmental screening system, which selects for well-adapted microbial communities. These environmental factors determine the habitat conditions and hence influence the seasonal community structure (Pernthaler et al. 1998). Nevertheless, an extensive microbial succession develops annually during a short ice-free period between July and October harboring organisms which are well suited to live under the fluctuating habitat conditions (Hinder et al. 1999a).

Temperature is a major environmental determinant affecting the abundance, productivity, and distribution of cold-adapted microorganisms. On the basis of their cardinal growth temperatures, extremophiles inhabiting cold environments are grouped as psychrophiles or psychrotolerants.

Traditionally, psychrophiles are defined as bacteria which have a T_{min} for growth below 0ºC, the T_{opt} below 15ºC, and the T_{max} below 20ºC (Morita 1975), whereas psychrotolerants (usually referred to as the psychrotrophs in the food industry), are defined as microorganisms which have a T_{min} for growth below 0ºC, the T_{opt} below 25ºC, and the T_{max} below 35ºC (Isaksen and Jørgensen 1996). More specifically, the psychrotolerants which show T_{opt} between 25 and 40 ºC and T_{max} between 35 to 40ºC are defined as mesophiles (Isaksen and Jørgensen 1996). Several authors have reported that many bacteria isolated from cold habitats are truly psychrophilic (Bowman et al. 1997; Staley and Gosink 1999) but a study by Franzmann (1996) also confirmed that isolates from Antarctica show a higher T_{opt} than the usual temperature of their environment. The majority of isolates from most low-temperature environments grow well at 0ºC to 4ºC, but they grow with higher rates as temperature increases. They are psychrotolerant rather than psychrophilic (Maruyama et al. 1997; Delille and Perret 1989). The studies mentioned so far, described phenotypic characteristics and to some degree the diversity of bacteria, which colonize cold habitats in general, but the adaptability of those bacteria to rapidly changing environmental conditions remains poorly studied.

Previous studies on microorganisms inhabiting cold and oligotrophic habitats including the Antarctic and Arctic region, permanently ice-covered seas, the deep sea, and mountain lakes have shown that heterotrophic bacteria, which were phylogenetically affiliated with the *Proteobacteria* and the *Bacteroidetes* (*Flavobacter-Cytophaga-Bacteroides-FCB*) group, represent a major population of these communities (Alfreider et al. 1996; Bahr et al. 1996; Hiorns et al. 1997; Bowman et al. 1997; Pernthaler et al. 1998; Ravenschlag et al. 1999; Wells and Deming 2003). The heterotrophic microbial processes play a central role in the pelagic food web through the utilization of dissolved and particulate organic matter (Sherr and Sherr 1994; Caron et al. 1997; Hinder et al. 1999b). In addition, microorganisms living in permanently cold ecosystems were found to contribute significantly to global carbon cycling, biomineralization and other biogeochemical processes (Skidmore et al. 2000; Sharp et al. 1999). Aerobic respiration with organic carbon as electron donor by metabolically active prokaryotes was found in a subglacial environment beneath a polythermal glacier (Skidmore et al. 2000) and it was suggested that these processes could drive chemical weathering beneath glacier and ice sheets (Sharp et al. 1999). Microorganisms inhabiting cold, high mountain lake habitats, however, are special compare to inhabitants of other extreme cold environments since they are well-adapted communities, colonizing habitats which are intensively exposed to fluctuating environmental conditions.

Recent investigations and discoveries of novel genera and species of cold and oligotrophic Antarctic sea environments has led to a deeper knowledge of autochthonous (indigenous) microbial communities in permanently-cold environments which are phylogenetically diverse (Bowman et al. 1997; Staley and Gosink 1999). Sequencing of 16S ribosomal RNA genes, fluorescent in-situ hybridization, and other molecular techniques have rapidly increased the knowledge about the diversity of bacteria and archaea present in many microbial habitats (Amann et al. 1990; Amann et al.

1995). So far, there is little information, however, about the phylogenetic diversity of heterotrophic, cold-adapted bacteria inhabiting low nutrient high-altitude lakes. Efforts in studying these microorganisms from diverse cold habitats might yield new bacterial strains with cold-active features and enzymes with a low affinity for substrates (Cavicchioli et al. 2002) and lead to a better understanding of physiological processes under oligotrophic, low temperature conditions.

Enriching for and isolating bacteria from oligotrophic low-temperature habitats involves the use of media which are poor in organic nutrients and incubation conditions designed to favor the growth of cold-adapted ecotypes. Boivin-Jahns et al. (1995) have compared bacteria using on one hand the classical phenotypic characterization including morphology, Gram staining, enzyme activities and the utilization of different organic substrates as carbon and energy sources as well as molecular analyses. They found that the phylogenetic analysis of small subunit rRNA gene sequences is more efficient for the identification of bacterial strains because misidentification of bacteria was less with the molecular methods. Employing the genotypic analysis and the characterization of communities has improved identification and it has led to the discovery of a number of possibly new species (Bowman et al. 1997; Sheridan et al. 2003).

We followed the hypothesis that microorganisms living in cold oligotrophic habitats had been selected for life at low temperature and low nutrient concentrations. They harbor physiological flexibilities to live under extreme and fluctuating conditions. Here we present evidence for their flexibility to adapt to two essential environmental determinants: temperature and nutrient concentration. The objectives of this study were to assess the diversity of cold-adapted heterotrophic bacteria and to study their ability to respond to changes in these two major environmental determinants. The study offers new insights into the microbial diversity inhabiting high altitude lakes.

2. Materials and methods

2.1. Description of study sites and sample treatment

Samples were collected from lakes located at slightly different altitudes within the Jöri catchment and the Tambo catchment. The catchment of the Jöri lakes is situated in the eastern Swiss Alps, (46º46’N / 9º58’E), at altitudes between 2'489 m and 3'060 m above sea level. The area is a rock desert, almost free of vegetation. Presently, there are 22 larger and small lakes within the Jöri catchment (Hinder et al. 1999a), some of them are several thousand years old, others formed when the glacier retreated during the last 150 years. The second site, the Tambo lake area, is located in south eastern Switzerland (46°20'N / 9°20'E); it contains 7 cascading glacial lakes at altitudes between 2'300 m and 2'350 m above sea level (Mez et al. 1998). Both areas contain remnants of glaciers whose meltwater feeds into some of the lakes directly. The samples for this investigation were obtained from

Jöri lakes I to XIV which have surface areas ranging from 700 m^2 (Jöri VIII) to 93'700 m^2 (Jöri I) and depths from 0.7 m (Jöri VIII) to 21.8 m (Jöri III) (Gabathuler 1999). The samples from Tambo were collected from lakes C, G, and F (Mez et al. 1998).

Lake water and biofilm samples were collected aseptically during the snow-free summer season. Subsurface water samples were obtained in sterile glass bottles and patches of biofilm samples were collected from the surface of submerged rocks. For the screening of oligotrophic and cold-adapted bacteria, 50 µl and 100 µl water samples were spread-plated and subcultured onto minimal growth agar medium (pH 7.1), whereas biofilm samples were spread aseptically onto the medium after mechanical disruption of the biofilm with a sterile loop. Growth temperature for all cultures was 4±1ºC. The minimal growth medium (designated as MM) contained the following ingredients (final concentration are given in µM if not described in more detail otherwise, final pH 7.1): $MgSO_4$ 6, $CaCl_2$ 10; Na_2CO_3 20; $NaNO_3$ 14; NH_4Cl 10; K_2HPO_4 1.75; EDTA Na salt 2.7; yeast extract 10 mg l^{-1}; peptone 10 mg l^{-1}, washed-bacteriological agar 15 g l^{-1} and trace element 1x, (added to the autoclaved basal medium). The concentrated trace element stock solution (10'000x) contained the following (mM): $ZnSO_4 \cdot 7H_2O$ 0.85; $MnCl_2 \cdot 4H_2O$ 7.1; $Co(NO_3)_2 \cdot 6H_2O$ 0.086; $Na_2MoO_4 \cdot 2H_2O$ 1.6; Citric acid·H_2O 29.75; and Ferric ammonium citrate 21.48.

Table 1. Origin of cold-adapted isolates, their codes and the identification numbers used in this study.

Isolate Origin	Isolate designations*)	Number of isolates
Jöri Lake I	J: **27, 28**, **31, 37, 43**, 50, 71, 77, 78, 84	10
Jöri Lake II	J: 09, **44**	2
Jöri Lake III	J: 01, 02, 04, 05, 06, 07, 47, 48, 49	9
Jöri Lake IV	J: **40**, 61, 64, 65	4
Jöri Lake V	J: 11, 20, **35**	3
Jöri Lake VII	J: 03, 14, 17, 18, 19**,** 45, 46, 51, 52, 53, 54, 55, 56, 59, 66, 67, 68, 79	18
Jöri Lake VIII	J: **38, 39**	2
Jöri Lake IX	J: 22, **29, 33**, 69	4
Jöri Lake X	**J: 32, 34, 41**, 76	4
Jöri Lake XI	J: 12, 13, 15, 16, 21, **36**, 73	7
Jöri Lake XII	J: **30, 42**	2
Jöri Lake XIII	J: 25, 26, 72, 75	4
Jöri Lake XIV	J: 10, 57,58, 60, 62, 63	6
Tambo C	T: 24	1
Tambo G	T: 23, 70, 74	3
Tambo F	T: 08	1
Jöri Glacier	J: 80, 81, 82, 83	4
TOTAL		84

*) Isolates which were originally from biofilm samples are written in bold letters, whereas planktonic and glacier isolates are written in plain letters.

When growth occurred (after 14 to 21 days of incubation in the cold), single colonies of visibly dominant and different colony morphotypes were subcultured onto new minimal medium. Isolates were maintained in 10 times diluted Luria Bertani (LB) agar medium (pH 7.2) containing the following ingredients: Bacto tryptone 0.5 g l^{-1}, yeast extract 1.0 g l^{-1}, NaCl 0.5 g l^{-1}, and bacteriological agar 15 g l^{-1}. Eighty four isolates were selected (Table 1) for further analysis and experimentation.

2.2. Genomic DNA extraction

Total genomic DNA from pure isolates was extracted either by employing the phenol extraction method (modified from Sambrook et al. 1989) or the CTAB (cetyltrimethyl ammonium bromide) method from 2.5 ml of cells grown to late log phase in 10 times diluted liquid LB medium. Cells were pelleted by centrifugation, the medium was decanted, and the pelleted cells were resuspended in 500 µl lysis buffer (50 mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 50 mM sucrose. Lysozyme was added to obtain a final concentration of 1 mg ml^{-1}, and the solution was incubated at room temperature for 10 min or until the solution cleared (complete cell lysis). 1% (v/v) of sodium dodecyl sulfate and 100 µg ml^{-1} of Proteinase-K were then added and the solution was incubated at 37ºC for 30 min and 55ºC for 10 min. The solution was chilled on ice and extracted with an equal volume of a phenol-chloroform-isoamyl alcohol (25:24:1) mixture. The aqueous phase was then washed twice with an equal volume of chloroform. Total genomic DNA was precipitated by the addition of 2.5 volumes of isopropanol followed by centrifugation for 10 to 20 min at room temperature. DNA was washed once with 70% ethanol and the pellet was then dissolved in 40 µl of double distilled water.

An alternative DNA extraction method with cetyltrimethyl ammonium bromide (CTAB) (modified from Murray and Thompson 1980) was also used. It provided a simple, less toxic and inexpensive method without the need for phenol extraction and yielded enough DNA template for PCR amplification. After pelleting the cells, extraction buffer (2% [w/v] CTAB, 100 mM Tris-HCl [pH 8.0], 1.4 M NaCl, 20 mM EDTA) was added and mixed well. The solution was incubated at 60ºC for 30 min and centrifuged at 13'000 rpm (±12'000 x g) (4ºC) for 10 min. After transferring the supernatant into a new tube, an equal volume of chloroform:isoamyl alcohol (24:1) was added, mixed gently and the solution was centrifugated at 13'000 rpm (±12'000 x g) (4ºC) for 10 min, these steps were repeated one more time. After centrifugation, the supernatant was transferred into a new tube and 1/10 volume equivalent of 7.5 M ammonium acetate was added. DNA was then collected by precipitation in ethanol. DNA extracts were checked using the following electrophoresis conditions: agarose gel (concentration 1 %; w/v), Tris-Acetate-EDTA (TAE) buffer 0.5X (20 mM Trizma base, 10 mM glacial acetic acid, 0.5 mM EDTA), running time 30 min at 5 V/cm. Successful extraction of DNA was verified by staining the gels in a 1 µg ml^{-1} ethidium bromide solution.

2.3. Amplification of 16S rRNA genes by PCR (Polymerase Chain Reaction) and screening by RFLP (Restriction Fragment Length Polymorphisms) analysis

Nearly full-lengths of the 16S rRNA genes were amplified by PCR using the bacterial primers: S-D-Bact-0008-b-S-20 (5'-AGA GTT TGA TCC TGG CTC AG-3') and S-D-Bact-1524-a-A-18 (5'-AAG GAG GTG ATC CAR CCG-3'). PCR amplification was performed in a 25 µl reaction volume

with a Techne Thermocycler (Techne LTD, Oxford Cambridge, U.K.). Each reaction mixture contained (final concentration) *Taq* buffer (1x) (Sigma), 1.5-2.0 mM $MgCl_2$, 0.1 mg ml^{-1} Bovine Serum Albumin DNase-free (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTP's, 200 nM of forward and reverse primer, respectively, 40 U ml^{-1} *Taq* Polymerase (Sigma), dH_2O and approximately 20-100 ng template DNA. The following touch-down PCR program was used: initial denaturation at 94ºC for 2 min, 20 cycles of 94ºC for 20 sec, 63ºC for 30 sec and lowering the temperature by 0.5ºC in every cycle, 72ºC for 80 sec; increasing the duration by 1 sec in every cycle, another 20 cycles of 94ºC for 20 sec, 53ºC for 30 sec, 72ºC for 100 sec; increasing the period by 1 sec every cycle followed by a final extension step at 72ºC for 10 min. PCR products were analyzed by electrophoresis in 1% agarose gels in 0.5x TAE running buffer, stained with ethidium bromide (1 µg ml^{-1}), and photographed under UV.

For RFLP analysis, 8 µl PCR products were double-digested with 1 U restriction enzyme *Hin*fI (5'-G/ANTC) and *Hae*III (5'-GG/CC) in a 10 µl total reaction volume. In a first approach, DNA fragments were separated using the following electrophoresis conditions: Agarose gel concentration 2%, Tris-Acetate-EDTA (TAE) buffer 0.5x, gel run for 60 min at 60 volts. 1 µl of digested DNA was loaded. The gels were stained with 1 µg ml^{-1} (final concentration) ethidium bromide solution. In a second approach, polyacrylamide gels (Spreadex EL800 Gel, Elchrom Sci.) were used with 30 mM Tris-Acetate-EDTA (TAE) buffer. The gels were run for 90 min at 80 volts (3.6 V/cm). 8 µl sample of a 10 µl total volume assay containing digested DNA and 5 µl of standard loading buffer were applied to each well of the gel. Detection of DNA bands was performed by staining the gels with 1 µg ml^{-1} ethidium bromide solution for 1 hour, and destaining with water for 45 min. The gels were photographed under UV transillumination.

2.4. TGGE (Temporal Temperature Gradient Gel Electrophoresis) analysis

A 1 µl volume of a 10^{-2} to 10^{-3} dilution of the PCR products was used as DNA template in a nested PCR to amplify an approximately 560 bp fragment using the bacterial primers S-D-Bact 341-b-S-17 (5'-CCT ACG GGA GGC AGC AG-3') and a GC-clamp (underlined) primer GC-Univ-907-a-A-20 (5'-<u>CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G</u> CCG TCA ATT CMT TTR AGT TT-3'). The PCR reaction was run under the following conditions: initial denaturation at 94ºC for 5 min, followed by 75ºC for 15 sec, 20 cycles of 94ºC for 20 sec, 65ºC lowered by 0.5ºC every cycle for 30 sec, and elongation temperature 72ºC for 1 min, followed by 15 cycles of 94ºC for 20 sec, 52ºC for 30 sec, 72ºC for 70 sec, and a final extension step at 72ºC for 10 min. TGGE was carried out with a DCode Mutation Detection system (Bio-Rad Laboratories). 10 µl of the PCR samples and 10 µl of the 2X loading buffer (70% [v/v] glycerol, 0.05% [w/v] bromophenol blue in water) were loaded onto 6% polyacrylamide gels (acrylamide: N,N'-methylene bis-acrylamide

37.5:1 [w/w]; 7 M urea, 1X TAE). The gels were run in the temperature range from 54ºC to 70ºC, temperature ramping rate was 1.1°C h^{-1}, voltage was 70V (3.5 V/cm), and running time was about 14 h. After the run, the gels were stained in 1 μg ml^{-1} ethidium bromide solution for 30 min, destained in water for 30 to 45 min, and photographed under UV.

2.5. Sequencing

The PCR products of the 16S rRNA genes from 19 isolates representing 15 different RFLP profiles (Fig. 2) were purified through microcon centrifugal filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA). Nearly full-lengths of the 16S rRNA genes were bidirectionally sequenced with a DNA sequencer (ABI Prism 377), using ABI Prism® Big Dye™ v2.0 (Applied Biosystems) as described by the manufacturer with the following 6 primers: S-D-Bact-0008-b-S-20 (5'-AGA GTT TGA TCC TGG CTC AG-3'), S-*-Univ-0519-a-A-18 (5'-GWA TTA CCG CGG CKG CTG-3'), S-*-Univ-0519-a-S-18 (5'-CAG CMG CCG CGG TAA TWC-3'), S-D-Bact-1099-b-S-16 (5'-GYA ACG AGC GCA ACC C-3'), S-D-Bact-1099-b-A-16 (5'-GGG TTG CGC TCG TTR C-3') and S-D-Bact-1524-a-A-18 (5'-AAG GAG GTG ATC CAR CCG-3'). For a 10 μl-single PCR reaction, 5 to 20 ng DNA template, 3 μl Big Dye (Applied Biosystems) and 3 μl of 1.5 μM primer were used. After the sequencing PCR, the products were purified with Sephadex G-50 (Amersham, Pharmacia Biotech AB) and loaded onto the sequencing machine (ABI Prism 377 DNA Sequencer).

2.6. Phylogenetic analyses

The BLAST Search tool available from NCBI (http://www.ncbi.nlm.nih.gov/blast) was used to list the closest neighbors of the sequences. The 16S rRNA gene sequences of the isolates and -if not already in the ARB databank- the closest neighbour sequences obtained from the BLAST Search were added into the ARB software environment (http://www.arb-home.de/). The new sequences were imported to the ARB database (release May 2002) and aligned automatically employing the Fast Aligner V1.03 of the ARB phylogeny software package. The alignment subsequently was corrected manually based on secondary structure information and the closest relatives as references. The sequences were added to the consensus tree that available in the ARB database and the distances were calculated using the maximum-parsimony approach. The overall phylogenetic affinity was evaluated using a consensus filter for all bacteria. For accurate phylogeny reconstruction the group specific filter was used. The phylogenetic trees were constructed by the neighbor-joining method as provided in the ARB package. The resulting neighbor-joining tree was evaluated by bootstrap analyses based on 100 resamplings.

2.7. Dendrogram based on virtual digestion and gel analyses

Virtual digestion using the custom digests program from New England Biolabs (NEB; homepage: http://www.neb.com/) of the 16S rRNA gene sequences obtained (ranging from positions 8 to 1524, *Escherichia coli* numbering) and real RFLP of amplified 16S rRNA genes (positions 8 to 1524, *E. coli* numbering) were used to build phylogenetic dendrograms. Fragments generated from restriction digests by *Hin*fI and *Hae*III restriction endonucleases were used for the clustering analysis. A binary data matrix was made based on the presence or absence of DNA fragments in the RFLP banding patterns. The distance matrix was calculated by Jaccard analysis (data not shown), from which a dendrogram was constructed by the neighbor joining method using the njPlot software program (Perriere and Gouy 1996).

2.8. Phenotypic characterizations of the isolates

2.8.1 Cell morphology by live-dead Gram staining

Fluorescent staining was performed on 19 isolates representing 15 different RFLP patterns to determine the Gram group, characterize cell morphology, and distinguish dead cells from living ones. Staining was carried out as described by the manufacturer (Molecular Probes, Inc.) using the Viability Gram Staining Kit (V-7023 Molecular Probes Inc.). The treated samples on slides were observed with a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen Germany) employing 3 different excitation filters 365-395 nm, 450-490 nm and 546-580 nm and photographed with an Optronic digital camera.

2.8.2 Determination of growth-temperature

For the preliminary study of the temperature preference of the isolates, 15 different isolates were spread on agar-solidified minimal medium (MM as described under 1), 10 fold diluted MM, 10 fold diluted Luria Bertani (LB) and 10,000 fold diluted LB. Identical plates inoculated with the same isolates were incubated aerobically at 3ºC, 8ºC, 12ºC, 16ºC, 24ºC, 30ºC, and 37ºC. For this preliminary study isolates of *E. coli* HB101*, Vibrio harveyi, Bacillus cereus* and *Pseudomonas aeruginosa,* which did not originate from oligotrophic habitats and which are not considered psychrophilic were used as reference strains.

Growth characteristics on MM were examined using 4 isolates J05, J71, J83, and J36 as representatives of the *alpha*, the *beta* and the *gamma* subgroups of *Proteobacteria* and the *Bacteroidetes* (*FCB*) group, respectively. Isolate of *E. coli* HB101 was used as reference strain. Prior to each experiment, these isolates were grown aerobically at 3±1ºC in 10 fold diluted LB broth

medium to the log-phase. Cells were aseptically washed 3 times by centrifugation, resuspended in new MM liquid medium, and 100 µl of the third suspension was used as inoculum for the following experiments. The inocula used had a cell density of about 10^3 to 10^4 cell ml^{-1}. Cells were cultivated in 75 ml flasks containing 25 ml MM broth medium and incubated aerobically at 3, 9, 15, 20, 24, 30, 37, and 42ºC. Growth at each temperature was determined periodically during incubation at the selected temperatures by direct counting, since the OD_{540} determination by spectrophotometer readings (absorbance values were between 0 and 0.003) did not reflect appropriate cell concentrations for the cultures at low-temperatures, as shown by the direct counting method. 10 µl of the culture were spread in an appropriate serial dilution onto solid MM medium. Plates were incubated at the selected temperatures, and visible colonies were counted after 3 weeks of incubation.

The growth characteristics in 10 fold diluted LB medium were examined using the same isolates. The isolates were pregrown aerobically at 3±1ºC in liquid MM until the log-phase was reached. These cultures were used as inocula with cell densities approximately 10^3 to 10^4 cells ml^{-1}. Growth experiments were carried out in 75 ml flasks containing 25 ml 10 fold diluted LB broth medium and incubated aerobically at 3, 9, 20, 30, 37 and 42ºC. 10 µl of the culture was used for direct counting by plating on solid 10 fold diluted LB medium. Plating was done in 24 hour intervals until the death phase was reached. The direct count method was used for all cultures because the isolates J71 and J83 rapidly produced an extracellular material, especially at temperatures above 20ºC, which influenced the OD determination. Plates were incubated at the appropriate temperatures, and visible colonies were counted after incubation periods of 1 week (at 3 and 9ºC), 2 or 3 days (at temperatures of 20ºC and above). *Escherichia coli* HB101 was used as reference strain. Colony forming units per milliliter were counted from plates containing 30 to 300 colonies. The dilution factors used were between 10^0 and 10^8. The maximum growth rate (μ_{max}) at each temperature was determined during the exponential phase.

2.8.3 Ice nucleation capability

Ice nucleation capability of all 84 isolates was tested qualitatively by the tube assay (modified from Hirano et al. 1985). Isolates were grown in 10 ml liquid MM and incubated at 4ºC until stationary phase was reached. Cells were then suspended in 10 ml autoclaved PBS solution (10 mM potassium phosphate-buffered saline, pH 7.0) and kept in a cooling-bath at -3ºC to -10ºC for 5 to 10 min. A suspension of *E. coli* cells containing plasmid pJL1703 (Loper and Lindow 1994) was used as positive control, whereas a cell-free PBS solution served as negative control.

3. Results

3.1 Temperature and nutrient fluctuation in high altitude lake habitats

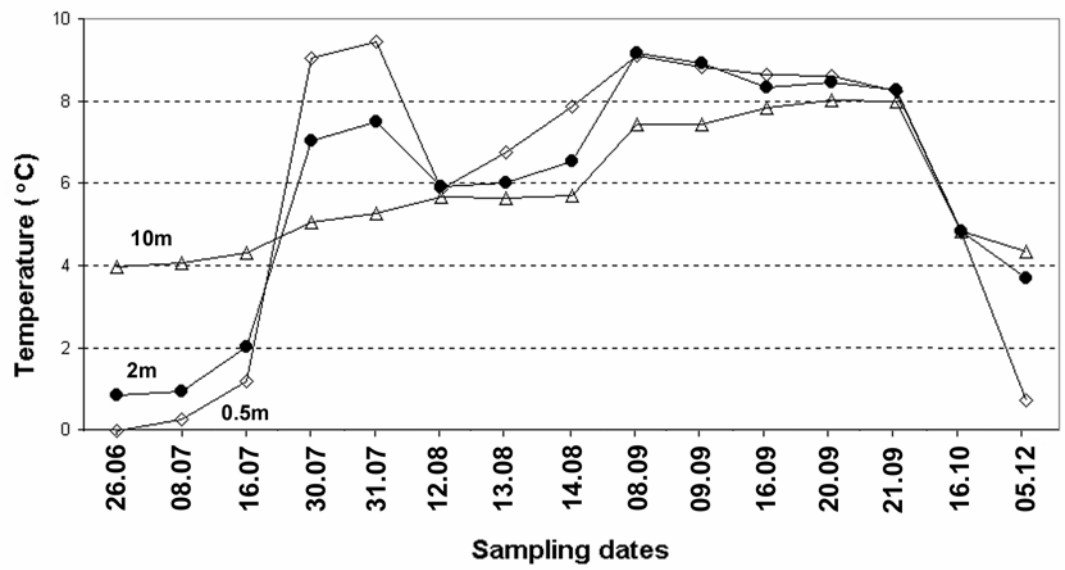

Figure 1a. Temperature fluctuation measured at 3 different depths: 0.5 m, 2 m, and 10 m in Lake Jöri XIII at the end of the ice covered period, several times during the summer season and at the beginning of the ice cover period in 2002.

Environmental temperature and nutrient concentrations are shown in Fig. 1a and Fig. 1b, respectively. These data demonstrate a high fluctuation of the two most influencial determinants, a physical and a trophic one, in high altitude lakes. Water temperatures measured in Lake Jöri XIII at the sampling dates indicated in Fig. 1a illustrate the extreme conditions, i.e. always below the optimal temperatures of the microorganisms isolated from these habitats. Near surface temperatures measured at 0.5 m depth showed the highest fluctuation (between 0°C to about 9.5°C). Temperatures at 2 m depth fluctuated less (1°C to 9°C), whereas temperature at the bottom of the lake showed the smallest fluctuations (4°C to 8°C). The data show periods when the lake was homothermic, indicating that the water masses were mixing. This happens in the middle of August at a temperature of 6°C and at the end of September to the middle of October at temperatures of about 8°C and 5°C.

Seasonal fluctuations in nutrient concentrations are shown by the ratio between N and P for Lake Jöri XIII (Fig. 1b). These two nutrients often become limiting for growth of the organisms in aquatic ecosystems and they are the ones with the most pronounced fluctuations. At the end of the ice cover period, N (sum of NH_4^+ and NO_3^-) showed the highest concentrations, which is due to the long dark period below the ice. NH_4^+ dominated at this time due to the low oxygen concentration which remained after the long winter period. The N/P ratio showed its highest values (around 200 over the

entire water column) during this period. In early summer, the N values decreased as a result of the consumption by autotrophic organisms. The P concentrations (measured as total dissolved phosphate in filtered samples) first increased due to its release from anoxic sediment and then decreases again as

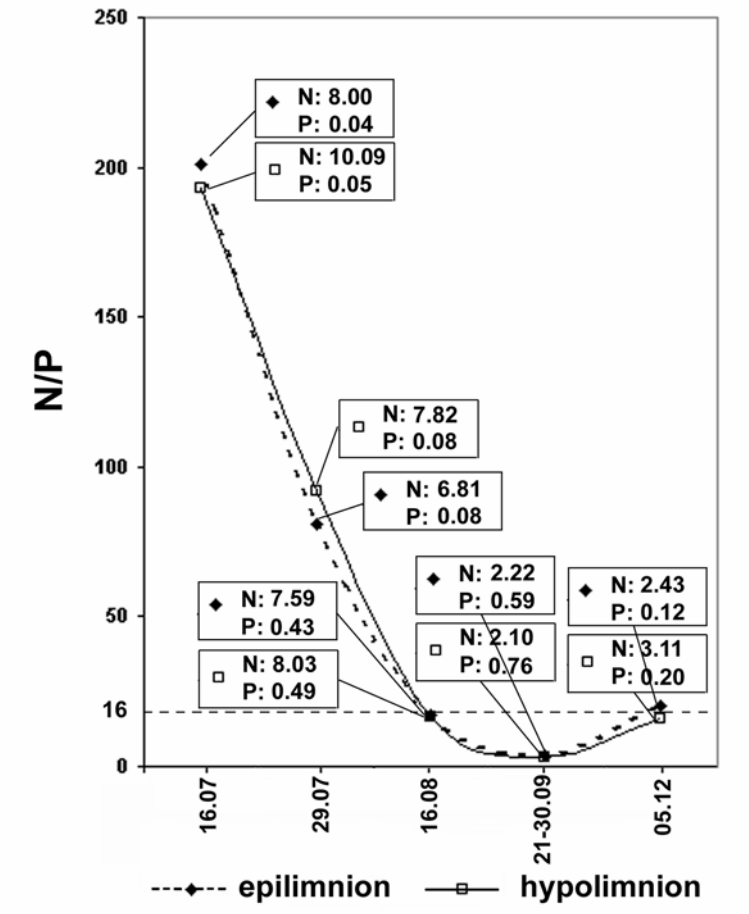

Figure 1b. Changes in nutrient conditions expressed by the ratio between N and P in Lake Jöri XIII, 2002. N/P is the classical Redfield ratio. N is the sum of dissolved NH_4^+ + NO_3^- (in μ mol/l) in filtered lake water. P is total dissolved phosphorous (inorganic and organic; in μ mol/l).

a consequence of high assimilation rates. In late summer, N concentrations were lowest, whereas PO_4 reached its maximum. The N/P ratios were thus lowest at the end of production period (between 3 in the epilimnion and 4 in the hypolimnion). Productivity slows down under the ice-cover, the lake becomes slowly anoxic in the hypolimnion, N is released from the sediment and the N/P ratio increases again.

3.2. PCR amplification of 16S-rRNA genes

Genomic DNA extracted by the phenol extraction or the CTAB method yielded good templates for further PCR amplification of the 16S rRNA gene. Amplifications with the primers S-D-Bact-0008-b-S-20 and S-D-Bact-1524-a-A-18 yielded products of approximately 1'500 bp, visible as single bands

on agarose gels. This size corresponds to the bacterial ribosomal small subunit RNA gene. Negative controls revealed no amplification products.

3.3. RFLP fingerprints of the isolates

RFLP analysis was used to screen isolates for unique fragmentation patterns. Amplification products of 84 isolates were treated with *Hae*III and *Hin*fI and the DNA fragments were separated by gel electrophoresis. Best separation was achieved with polyacrylamide Spreadex EL800 (Elchrom Scientific) gels, which allows one to separate DNA sizes ranging from about 50 bp to 400 bp (Fig. 2).

Isolates, whose RFLP patterns showed many fragments of similar sizes and only a few different one, i.e J62 and J35; T70 and J06; J36 and J37 (Fig. 2) represent different isolates and indicate that genotypically they are closely related. As will be shown later, sequence analyses revealed that DNA fragments with similar RFLP patterns were phylogenetically closely related.

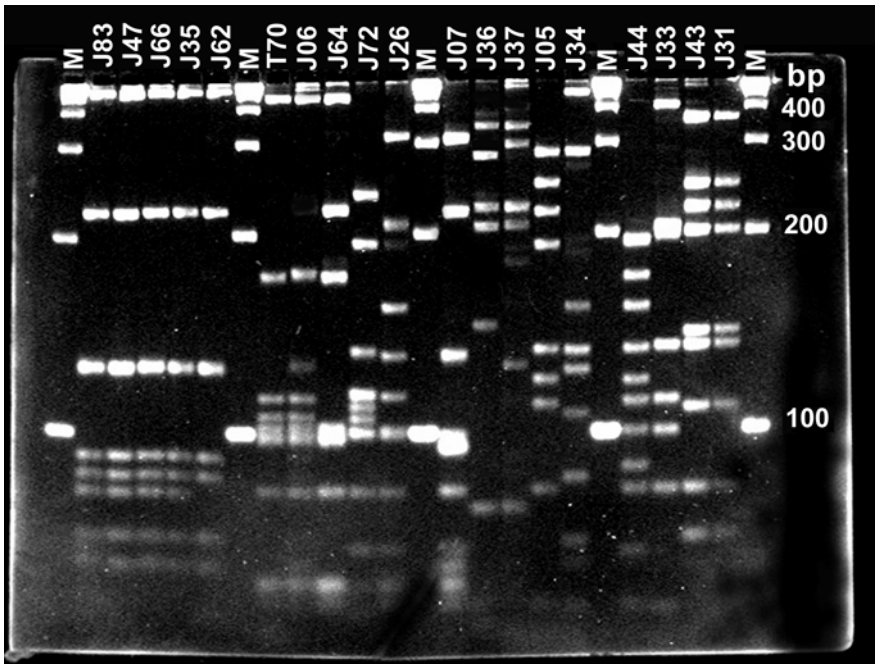

Figure 2. Ethidium bromide-stained gel showing separation patterns of bacterial DNA encoding 16S-rRNA genes from the isolates. J83 shows corresponding band patterns with isolates J47, J66 and J35; J43 has a corresponding pattern to J31. M: molecular marker 100 bp ladder (Pharmacia, Biotech). Electrophoresis conditions for Spreadex EL-800 gel (Elchrom Sci.): 70 Vcm^{-2} for 90 minutes, in 30 mM TAE buffer.

Fifteen unique RFLP patterns were obtained from 84 isolates. The pattern represented by isolate J83 was found in 32 isolates (38.1 % out of 84 isolates analyzed) and the pattern of J43 was observed in 30 isolates (35.7 %), respectively (Table 2). Some RFLP patterns occured in only one bacterium isolated from biofilms, i.e RFLP pattern no. 3 (Table 2) represented by J33 (Fig. 2), pattern no. 5 represented by J34, pattern no. 6 represented by J44, and patterns no. 8 and 9 represented by J36 and

J37, respectively. Some RFLP patterns were found in planktonic isolates but never in biofilm-associated isolates (e.g. RFLP patterns no. 2, 7, 11, 10, 12, 13, 14 and 15 represented by J62, J05, T70, J06, J64, J72, J07 and J26 respectively). The RFLP patterns represented by J83 and J43 were common for planktonic and biofilm-associated bacteria. Isolates originating from Tambo lakes revealed one unique RFLP pattern, represented by T70; whereas the patterns of T08, T23, and T24 were the same as in the group represented by J43, originating from the Jöri lakes.

Table 2. Distribution of RFLP patterns of the 16S rRNA genes of all isolates digested by *Hae*III and *Hin*fI, their phylogenetic affiliation and average G+C content as calculated from the 16S-rDNA nucleotide sequences.

Pattern No.	Isolates with the same RFLP patterns*)	Number of isolates and % of total	Phylogenetic group as confirmed by DNA sequences	G+C content (%) in the 16S rDNA
1.	01, 03, 13, 16, 17, 18, 20, 21, **27b, 28b, 35b, 38b, 39b, 40b, 41b**, 47, 48, 50, 51, 52, 54, 58,59, 60, 61, 66, 67, 68, 69, 79, **80g, 83g**	**32 (38.1%)**	Gamma-Proteobacteria	
2	62	**1 (1.1%)**	Gamma-Proteobacteria	**53.6 ±0.1**[1)]
Total of *Gamma-Proteobacteria*		**33 (39.2 %)**		
3.	**29b, 30b, 33b**	**3 (3.6 %)**	Beta-Proteobacteria	
4.	T08, 09, 10, 12, 14, 15, 19, 22, T23, T24, 25, **31b, 42b, 43b,** 45, 46, 49, 53, 55, 56, 57, 63, 71, 73, 76, 77, 78, **81g, 82g,** 84	**30 (35.7 %)**	Beta-Proteobacteria	**53.3±0.1**[2)]
Total of *Beta-Proteobacteria*		**33 (39.3 %)**		
5.	**32b, 34b**	**2 (2.4%)**	Alpha-Proteobacteria	
6.	**44b**	**1 (1.1 %)**	Alpha-Proteobacteria	
7.	04, 05	**2 (2.4 %)**	Alpha-Proteobacteria	**55.0±0.1**[3)]
Total of *Alpha-Proteobacteria*		**5 (5.9%)**		
8.	**37b**	**1 (1.2 %)**	Flavobacterium-Cytophaga-Bacteroides (FCB)	
9.	**36b**	**1 (1.2 %)**	Flavobacterium-Cytophaga-Bacteroides (FCB)	**51.0±0.1**[4)]
Total of *Flavobacterium-Cytophaga-Bacteroides*		**2 (2.4%)**		
10	06	**1 (1.2%)**	Actinobacteria	
11.	T70, T74	**2 (2.4 %)**	Actinobacteria	
12.	64, 65	**2 (2.4 %)**	Actinobacteria	
13.	72	**1 (1.2 %)**	Actinobacteria	
14.	02, 07	**2 (2.4 %)**	Actinobacteria	
15..	11, 26, 75	**3 (3.6 %)**	Actinobacteria	**56.3±0.1**[5)]
Total of *Actinobacteria*		**11 (13.2%)**		
Total		**84 (100 %)**		

*)All isolates without letter designation are planktonic samples from Jöri lakes, **g** designates glacier samples, **b** designates biofilm samples, and **T** designates isolates from Tambo lakes.
[1)] Average G+C content of 16S rRNA genes of isolates J35, J47, J62, J66, and J83.
[2)] Average G+C content of 16S rRNA genes of isolates J31, J33, and J43.
[3)] Average G+C content of 16S rRNA genes of isolates J05, J34, and J44.
[4)] Average G+C content of 16S rRNA genes of isolates J36 and J37.
[5)] Average G+C content of 16S rRNA genes of isolates J06, J07, J26, J64, J72, and T70.

3.4. TTGE fingerprint of the isolates

For TTGE, approximately 560 bp fragments of the 16S rRNA genes which were amplified by the S-D-Bact-341-b-S-17 and GC-Univ-907-a-A-20 primer set were used. TTGE analysis was also used to analyze the purity of the amplified 16S rRNA genes as revealed by a single band in the gel for each

isolate (Fig. 3). This method can be used to determine differences of 16S rRNA gene sequences among the isolates, which cannot be distinguished by RFLP patterns. Isolates J83, J47, J66, and J35, which reveal identical RFLP patterns (Fig. 2) can clearly be distinguished as 2 discrete patterns on TTGE gels (Fig. 3).

It can also be observed that discrete RFLP patterns can give the same TTGE banding pattern, for instance patterns for isolates J06 and J64, J34 and J44, J36 and J37, J31 and J43. They have identical migration distances on TTGE gels since they have very small differences (0 to 3 bases) in their G+C content within the 560 bp fragment of the rRNA genes amplified by the TTGE primer sets. Their TTGE banding pattern allows one to distinguish them into different groups. Sequencing of the 16S rRNA genes confirmed that the DNA of TTGE bands which show the longest migration distances belong to the same group of *Actinobacteria* (*Gram positive, High G+C bacteria*), i.e. T70, J06, J64, J72, J26, and J07. The average G+C content of their 16S rRNA genes was 56.3%± 0.1 (Table 2) which leads to DNAs with high melting temperatures (T_m).

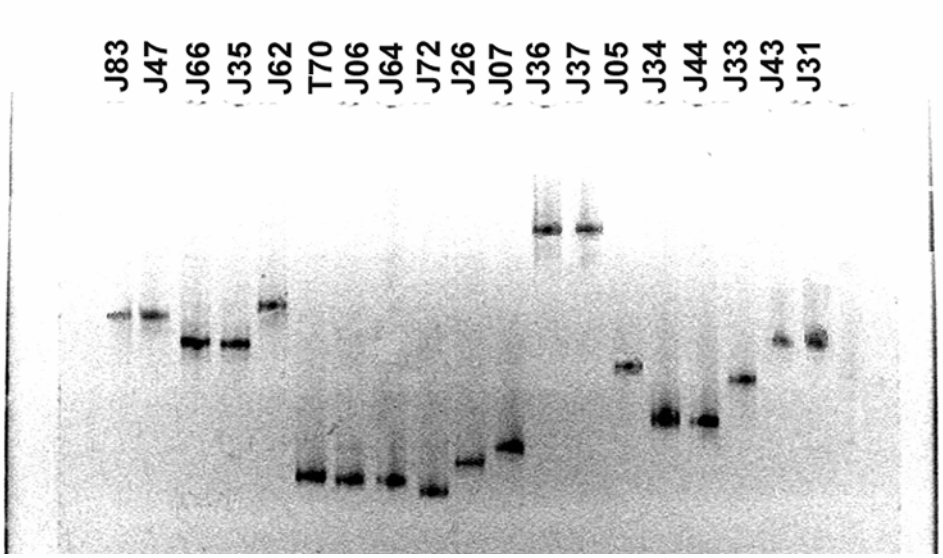

Figure 3. Ethidium bromide-stained acrylamide gel showing TTGE separation pattern of bacterial DNA encoding 16S-rRNA genes from the isolates. Running conditions: 80 V, initial temperature 54ºC, final temperature 70ºC, temperature ramp: 1.6ºC h^{-1}, running time 12 h.

3.5. Phylogenetic affiliation of the isolates

16S rRNA genes from representative isolates expressing unique RFLP patterns were selected to study the phylogenetic relationship. Both strands of almost full-length 16S rRNA gene sequences with sizes ranging from 1441 to 1498 bp were sequenced. Detailed phylogenetic positioning of the representative strains is depicted in Fig. 4.

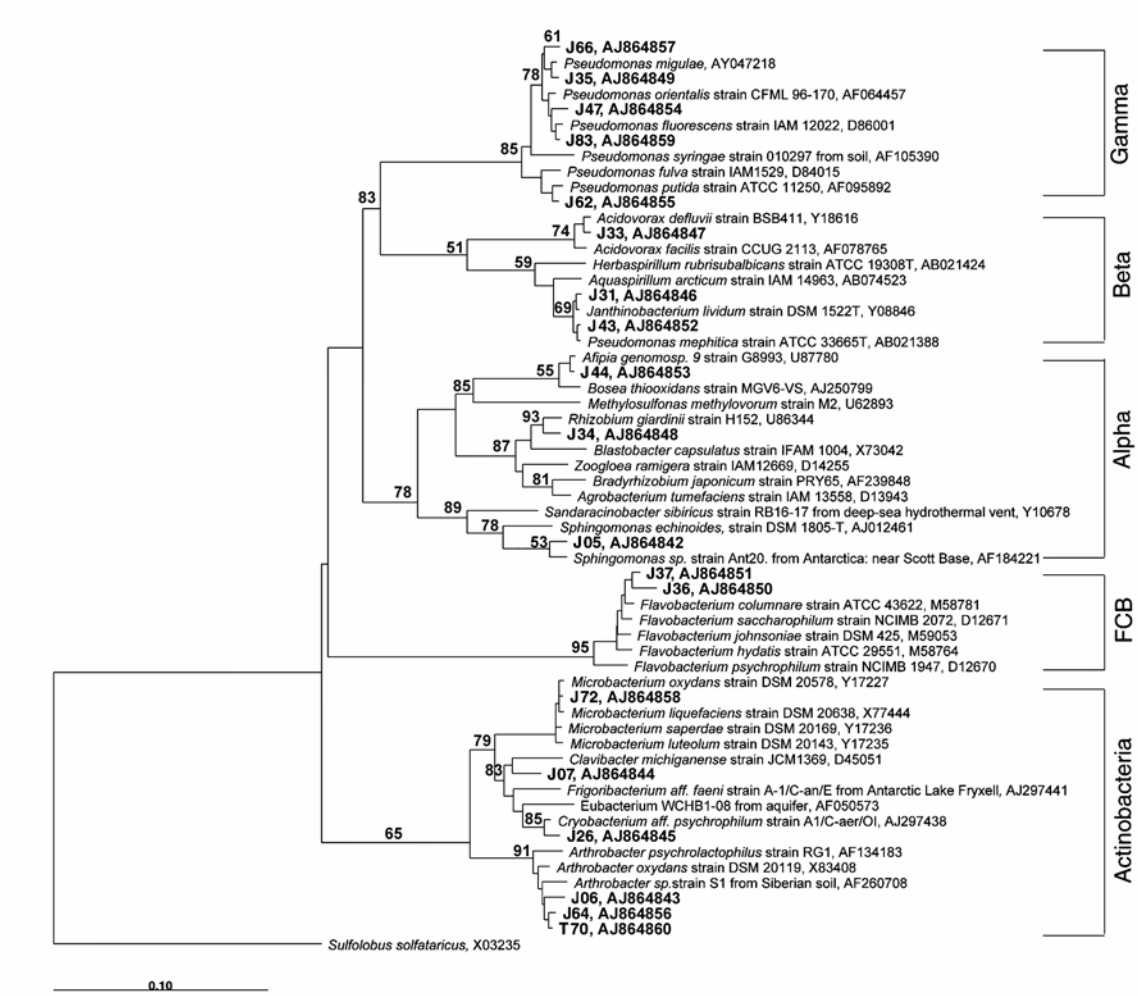

Figure 4. Phylogenetic affiliation of 16S rRNA gene sequences of isolates from cold, oligotrophic high mountain lakes at Jöri and Tambo. Names of the isolates designated by prefix J (Jöri) or T (Tambo) are listed and written in bold, followed by the accession numbers with the closest neighbors written in italics. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The tree is rooted using *Sulfolobus solfataricus* (X03235) as a representative of the outgroup *Archaea* (Phylum *Crenarchaeota*). The scale bar indicates 10% calculated sequence difference.

The 16S rRNA gene sequences obtained group into five bacterial phylogenetic branches, i.e the *alpha*-, *beta*- and *gamma-Proteobacteria*, the *Actinobacteria* and the *Bacteroidetes* (*FCB*) group. All of the isolates, which are affiliated with the *Proteobacteria* group belong to the Gram negative bacteria. The *alpha-Proteobacteria* (5.9%) group falls into the families Sphingomonadaceae, Bradyrhizobiaceae and Rhizobiaceae. Isolate J05 is closely related to the family Sphingomonadaceae (i.e *Sphingomonas* sp.strain Ant20, originating from Antarctica). J05 is a planktonic, yellow-pigmented, and rod shaped cells. The biofilm-associated isolates J44 and J34 are short rods. They are closely related with the genus *Afipia genomospecies* (family Bradyrhizobiaceae) and *Rhizobium giardinii* strain H152 (family Rhizobiaceae) which were not found in permanently cold environments before. These genera are well known as symbiotic microorganisms belonging to the nitrogen-fixing bacteria, which grow under microaerophilic conditions (Jordan 1984 *In* Krieg and Holt, 1984).

Over one half of the cultivable heterotrophic bacteria from these habitats are closely related to the *beta*- and *gamma-Proteobacteria*, which were represented by 33 isolates (39.2%) of each. Isolates from the *beta*-subdivison were originally isolated from water samples as planktonic or biofilm-associated bacteria, and from glacier ice. They fall into the family Oxalobacteriaceae and Comamonadaceae. The closest relative of the Gram negative, long rod shaped cells of J43 is *Pseudomonas mephitica,* and the next relative of J31 is *Janthinobacterium lividum* (both belong to the family Oxalobacteriaceae). The rod shaped cells of J33 are clustered within *Acidovorax defluvii*, which belongs to the family Comamonadaceae. As many as 5 isolates from water samples which branch closest to *Pseudomonas mephitica*, i.e. isolate J43, J71, J77, J78, and J84 are able to induce ice formation in the test tube assay (data not shown). To our knowledge, this is the first report of the ice formation capability found in bacteria inhabiting high altitude lakes.

Thirty three isolates (39.2%) fall into the *gamma*-subdivison of the *Proteobacteria*. They were strains isolated from biofilms, water samples, and glacier ice. These strains are related to the family Pseudomonadaceae, i.e *Pseudomonas migulae, P. fluorescens,* and *P. putida.* They belong to the Gram negative bacteria, are short rods to rod shaped cells. Generally they are cream-pigmented morphotypes, and most of them were capable of producing water-soluble yellow-greenish fluorescent pigments. One member of this group was able to catalyze ice formation, i. e. isolate J83, which originated from a glacier sample.

The RFLP analysis from the members of the Gram positive isolates showed that genotypically and phylogenetically they are diverse compared to other groups. These isolates were affiliated with the *Actinobacteria* group (13.2%), were planktonic, and showed diverse cell shapes from short to discus shaped, rods, or cocci. Three isolates belong to the family Microbacteriaceae, i.e J26, J07, and J72 whose closest neighbors are *Cryobacterium psychrophilum, Clavibacter michiganense,* and *Microbacterium liquifaciens*. Three other isolates with rod or cocci like morphotypes were yellow or orange-pigmented (T70, J64, J06) and belong to the family Micrococcaceae, i.e *Arthrobacter* sp. These genera are not spore-forming, obligately aerobic bacteria, are commonly found in soil, and have diverse cell shapes, involving conversion from rod to sphere, coccoidal and back to rod again during growth in complex media (Keddie et al. 1986 *In* Sneath et al. 1986).

The 16S rRNA gene sequences of the non-pigmented, biofilm-associated, motile, rod-shaped cells of the isolates J36 and J37 fell into the *Bacteroidetes* (*FCB*) group (2.4%). They were affiliated with *Flavobacterium columnare*. This result supports the previous report that the *FCB* group was commonly found in aquatic habitats, both freshwater and marine (Staley and Gosink 1999) and many genera of the *FCB* group are capable of growing below 20ºC.

Sequences with similarity values ≥97% are considered to belong to known species or sequences of clones present in the databank. Most of our isolates were found to be closely related to known sequences of microorganisms present in the GenBank as indicated by the shallow branches in the

phylogenetic tree. Sequences revealing similarity values <97% when compared with known isolates can be considered as novel species (Stackebrandt and Goebel 1994; Pinhassi et al. 1997) i.e. isolate J07 which showed only 96.7% similarity with *Clavibacter michiganense* strain JCM 1369.

3.6. Comparative cluster analysis of RFLP patterns

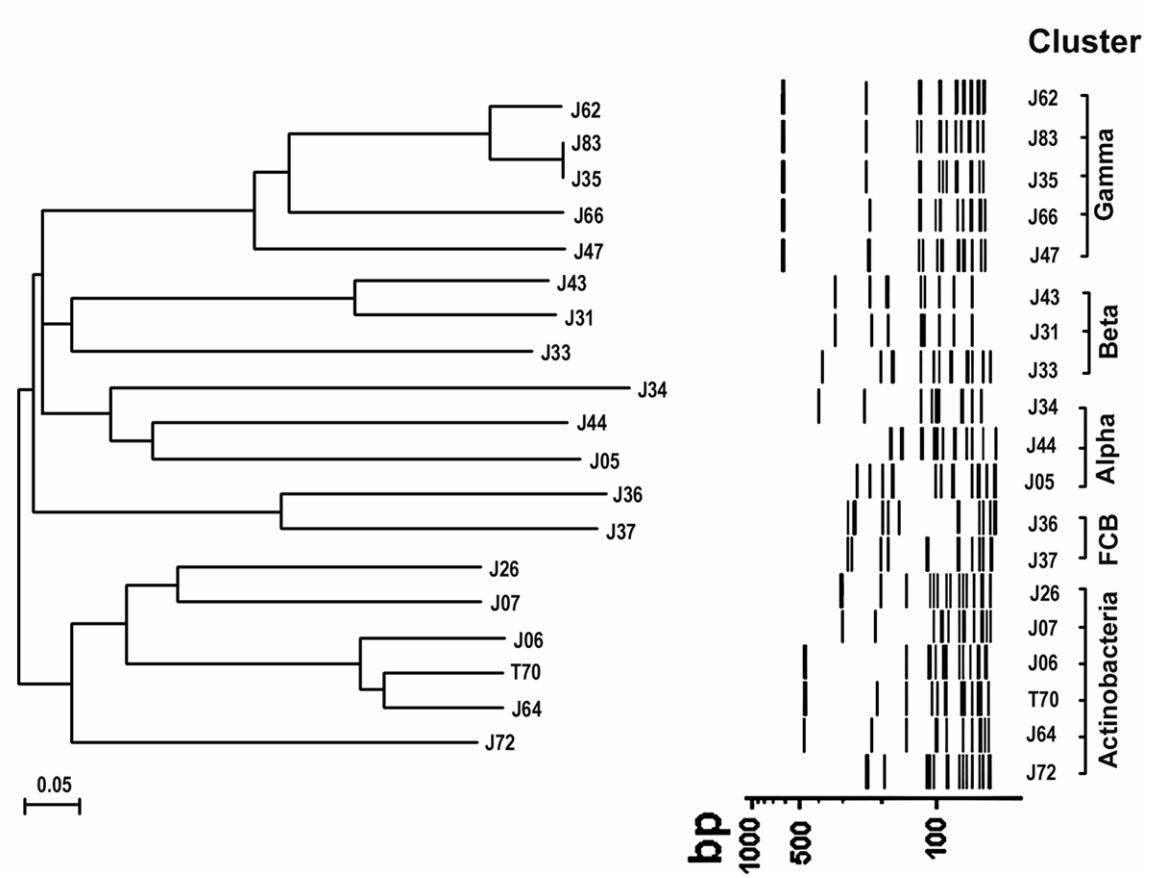

Figure 5. Cluster analysis (left) based on virtual digestion of 16S rRNA gene sequences of the isolates (named on the right) employing *Hinf*I and *Hae*III with the *Actinobacteria* as an outgroup. The scale bar indicates 5 % estimated banding pattern dissimilarity.

Cluster analysis approach was used to test the reliability of RFLP to preselect different organisms and to obtain a rough overview of the diversity before sequencing. We constructed dendrograms with the virtual and the real patterns and searched for topology similarities. Cluster analysis of virtual digestion patterns generated from 16S rRNA gene sequences data from the isolates correspond fairly well to the phylogenetic results derived from analysis of the actual 16S rRNA base sequences. The dendrogram obtained show five different clusters (Fig. 5) i.e. an *FCB* cluster represented by J36 and J37; an *alpha-Proteobacteria* cluster represented by J34, J44, and J05; an *Actinobacteria* cluster

represented by J26, J07, J06, T70, J64, and J72; a *beta-Proteobacteria* cluster represented by J43, J31, and J33, and a *gamma-Proteobacteria* cluster represented by J62, J83, J35, J66, and J47. Isolates which share a large number of coresponding DNA fragments (bands in the two different RFLP analyses) belong to the same cluster. The similarities between the two RFLP analyses are striking, and they do not contradict the clustering based on the sequence analyses. The similarity values obtained from the RFLP analyses were less than those obtained for the 16S rRNA gene sequence analyses, as indicated by the deep branch lengths. This is due to the lower number of characters (DNA bands) which were available for the RFLP analysis.

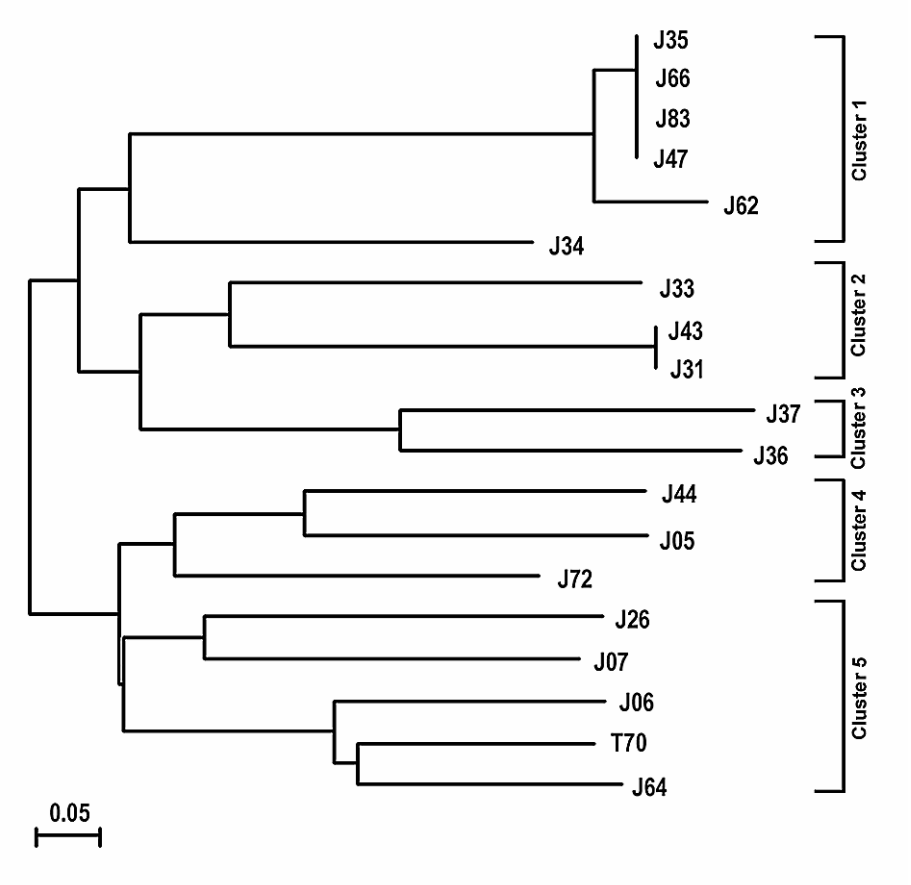

Figure 6. Cluster analysis based on real gel analysis (see Fig. 2) of PCR amplified 16S rRNA gene fragments of the isolates with *Hinf*I and *Hae*III, with the *Actinobacteria* as an outgroup. The scale bar indicates 5 % estimated banding pattern dissimilarity.

The patterns generated by the gel image of the RFLP bands in the gel analysis resulted in a slightly different clustering as compared to that of the virtual RFLP derived from analyses of the actual 16S rRNA base sequences. For instance, the RFLP of J44 and J05 fell into the cluster with J72, instead of clustering with J34 (Fig. 6) as it does in the virtual digest. The differences are due to the much lower

number of characters (DNA bands), which could be used for the real RFLP analysis compared to the virtual digestion analysis. The number of distinguishable characters used in the gel analysis was 41, while there were 104 characters available in the virtual digestion. In the gel image, bands with sizes larger than 400 bp and very small fragments (< 50bp) were not separated distantly enough, which makes it difficult to distinguish them as distinct characters.

3.7. Growth temperature studies

Our preliminary study on growth experiments showed that none of the isolate could be considered an obligate psychrophile, as defined by Morita (1975). Isolates which were affiliated with the *alpha–Proteobacteria* group, the *FCB* group and the *HGC* group were able to grow at temperatures between 3 ± 1ºC and 24ºC; but no growth on MM medium was observed at 30ºC. As expected, the growth rate ratios varied considerably (Fig. 7). Isolates belonging to the *beta* and *gamma* subgroups of the *Proteobacteria* showed a broader range of temperature tolerances, ranging from 3 ± 1ºC up to 37ºC on all types of low-nutrient media. None of the reference strains was able to grow at temperatures lower than 10ºC on any of the media used, except *B. cereus* which grew at a minimum temperature of 9ºC. At higher temperatures (tested up to 37°C), *V. harveyi, P. aeruginose* and *E. coli* grew best on 10 fold diluted LB, less on MM, and poorly on 10 fold diluted MM and 10,000 fold diluted LB media, whereas *B. cereus* was able to grow on all types of low-nutrient media.

The maximum growth rate (μ_{max}) ratios compared to *E. coli* were measured for the isolates J05, J71, J83 and J36 grown in MM and 10 fold diluted LB and at various temperatures of incubation (Fig. 7). All representative isolates showed growth which classifies them as psychrotolerant or mesophilic bacteria. All isolates were able to grow at the lowest temperatures tested and the nutrient concentration played minor roles at this temperature. At higher temperatures, the μ_{max} differences between the isolates grown in MM and 10 fold diluted LB medium were more pronounced than those measured at lower temperatures. All isolates showed lower μ_{max} values when they were grown in MM than when grown in 10 fold diluted LB medium. Isolates J05 and J36 showed a narrower temperature range than isolates J71 and J83. Based on their optimum temperature, our isolates can be grouped into two psychrotolerant groups: isolates J05 (despite of its ability to grow at 30ºC on 10 fold diluted LB medium) and J36, which have their optimum temperature below 25ºC, can be grouped as psychrotolerant bacteria, whereas the isolates J71 and J83 are mesophiles (Isaksen and Jørgensen 1996).

In MM, the optimum temperature of the isolate J05 was about 20ºC ($\mu_{max,}$ 0.09 h^{-1}) and the optimum temperature for the isolate J36 ranged from 9 to 20ºC ($\mu_{max,}$ 0.07 h^{-1}). Both isolates showed a lower μ_{max} at 24ºC, i.e 0.08 h^{-1} and 0.05 h^{-1}, respectively. At 30ºC, these isolates did not grow.

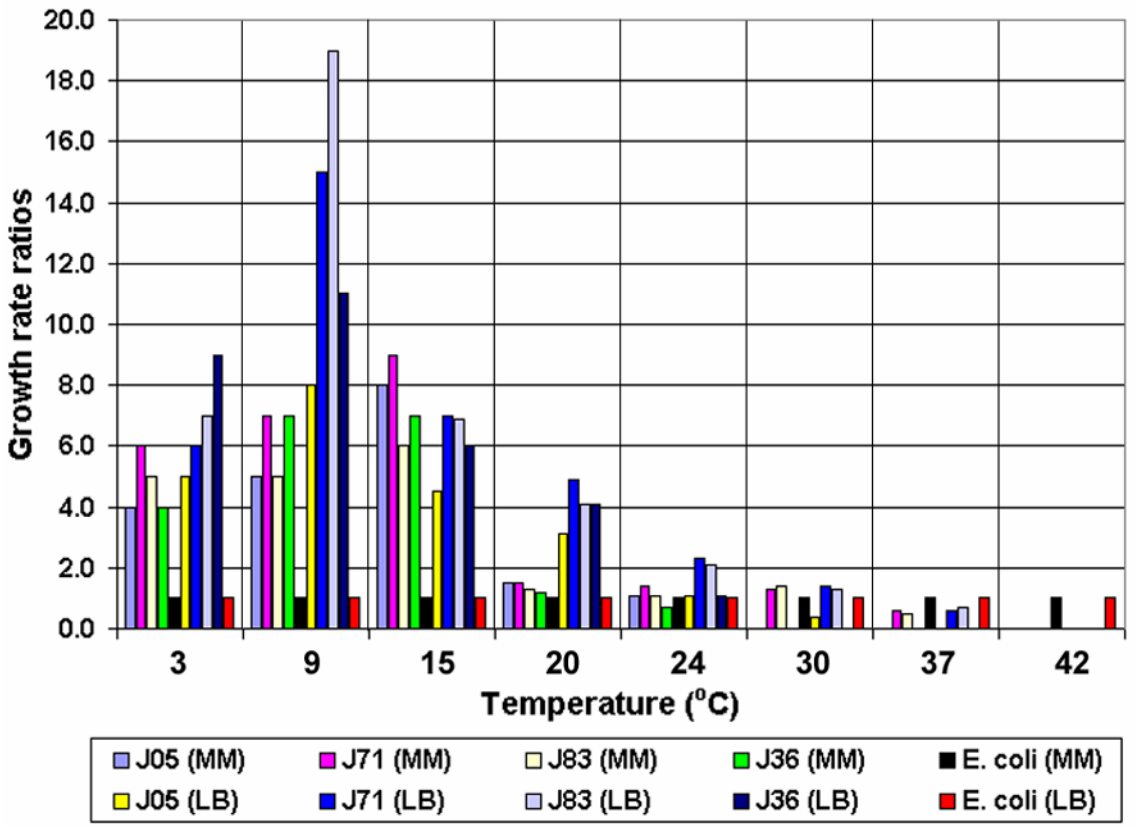

Figure 7. Growth rate ratios of the isolates grown in MM and 10-fold diluted LB medium at different temperatures compared to the corresponding growth rate of *E. coli**).

*)Growth rate ratios at 3°C show the minimum values because *E. coli* colonies did not grow at this temperature. Growth rate values used for this temperature were compared to those of *E. coli* incubated at 9°C.

Isolates J71 and J83 exhibited higher optimum temperatures for growth compared to the isolates J05 and J36. The isolate J71 grew optimally at temperatures ranging from 24 to 30ºC ($\mu_{max,}$ 0.10 h^{-1}), whereas the optimum temperature of the isolate J83 was about 30ºC ($\mu_{max,}$ 0.11 h^{-1}). A mesophilic reference strain of *E. coli* HB101 had an optimum temperature of about 37ºC ($\mu_{max,}$ 0.09 h^{-1}).

All isolates showed higher μ_{max} values when they were grown in the medium with higher nutrient concentration (10 fold diluted LB medium), especially at the incubation temperatures higher than 3ºC. The optimum temperatures of the isolates J05 and J36 were about 20ºC, with μ_{max} values of 0.22 h^{-1} and 0.29 h^{-1}, respectively. In this medium, the isolate J05 was able to grow although with a lower μ_{max} value at 30ºC ($\mu_{max,}$ 0.10 h^{-1}), compared with μ_{max} at 20ºC ($\mu_{max,}$ 0.22 h^{-1}), whereas J36 which showed the highest μ_{max} at 3ºC, did not grow at all at 20ºC. Both isolates J71 and J83 showed the highest μ_{max} values of 0.36 h^{-1} and 0.34 h^{-1}, respectively, at their optimum temperatures of approximately 30ºC; they did not grow at 43°C any more. *E. coli* HB101 had an optimum temperature of about 37ºC ($\mu_{max,}$ 0.34 h^{-1}).

4. Discussion

Oligotrophic, low temperature high mountain lake habitats are good models for microbial ecosystem studies. The two ecological determinants select for communities whose members must be able to tolerate the harsh conditions and to respond to fluctuations. Seasonal environmental fluctuations provide physical and trophic heterogeinities which strongly influence community diversity. We addressed questions related to the microbial diversity in these habitats, and the physiological flexibility of some of the member populations towards environmental changes.

We used culture-dependent molecular techniques (PCR-based analysis of bacterial isolates) to assess the diversity and culture-independent approaches and to identify microorganisms. The PCR-RFLP method in combination with 16S-rDNA sequencing analyses are powerful tools to identify populations and to provide information about community complexity. The phenotype characterization, on the other hand, allows one to identify bacteria to the genus level (Bowman et al. 1997) thereby adding physiological information to the molecular one.

Previous study (Prüβ et al. 1999) reported that 16S-rRNA gene sequence information allowed to distinguish the rDNA signatures between psychrotolerant and mesophilic strains from the same genus of *Bacillus* sp.. Based on their 16S rDNA base sequences between 180 to 192 (*E. coli* position), the mesophilic strains of *B. cereus* and *B. thuringiensis* possess the following sequences: AA**C** ATT TTG AAC **C**GC AT**G** GTT C, whereas the psychrotolerant strains of *B. mycoides* and *B. weihenstephanensis* revealed the following bases: AA**T** ATT TTG AAC **T**GC AT**A** GTT C (Prüβ et al. 1999). In our study, the 16S rDNA signatures apparently cannot be used to dinguish the mesophilic strains (J83: ACG TCC TAC GGG AGA AAG CAG G and J71: ACG ATC TAA GGA TGA AAG TGG G) from the psychrotolerant ones (J05: GAT GAC GTA AGT CCA AAG ATT T and J36: AGC ATT GCA GAA TGG CAT CAT T). This is may be due to the high sequence variability of different genera in these bases positions.

The culture-based method provides possibilities for characterizing genotypes and phenotype properties of bacteria from various habitats, and furthermore for experimentally studying their roles in the environment. This method is limited, however, by potential selection during enrichment and isolation procedures. Previous attemps in culturing bacteria from permanently cold sea ice at subzero temperatures revealed that the proportion of the community which escaped cultivation, the so-called enumeration anomaly (Staley and Gosink 1999), typically ranged from 38 to 99% (Junge et al. 2002). For ocean samples (Amman et al. 1995) reported an enumeration anomaly of ± 0.01%. With culture-dependent methods one often obtain isolates belonging to a relatively narrow genotyphic diversity. Our 84 isolates represent 15 unique RFLP patterns, which fall into 5 different major phylogenetic groups. In contrast, 75 biofilm-associated bacterial clones obtained from the Lake Jöri XIII habitat revealed 38 different RFLP profiles, which fell into 9 major phylogenetic groups belonging to 20 genera (Yuhana, unpublished).

The majority of our isolates fell into the *beta* and *gamma* subgroups of the *Proteobacteria* (39.5% each). *Beta-Proteobacteria* appeared to dominate freshwater bacterial communities (Glöckner et al. 1996; Alfreider et al. 1996; Pernthaler et al. 1998; Glöckner et al. 2000; Brümmer et al. 2003), whereas *gamma-Proteobacteria*, which are abundant in marine habitats (Cho and Giovannoni 2004), are found less frequently in high mountain and freshwater habitats (Alfreider et al. 1996; Glöckner et al. 2000). The results might be influenced by the culture-dependent selection, or by the high abundance of these groups during the sampling periods. Wagner et al. (1993) using culture-dependent approaches, were able to demonstrate shifts in the microbial structure of activated sludge. They also reported that nutrient-rich media (LB) favored the growth of the *gamma-Proteobacteria*. Our results show that the *beta-* and the *gamma-Proteobacteria* rapidly responded and grew well, and were therefore selected, even in nutrien-poor media.

The observations imply that the heterotrophic cold-water isolates from lake habitats are able to adapt their growth rate to the enormously variable temperature conditions and nutrient concentrations which are common in this habitat. The results of the growth experiments showed high flexibility and adaptability of the isolates belonging to these subgroups to grow at higher temperatures and nutrient concentrations. During the summer season, the time in which those isolates were collected, mountain lakes undergo changes in their trophic levels from oligotrophic to mesotrophic (Iqbal-Nava 2003) whereas the water temperature can fluctuate by ± 10ºC in a few days. The results of the growth experiments suggest that several representatives of the *Proteobacteria* subgroup were able to maintain large cell numbers and thus predominate in the bacterial community of these habitats.

Maximal growth rate values and lag states (the time before the cell division is initiated) might determine the psychrotolerant competitiveness in an environment possessing the fluctuations observed (Rutter and Nedwell 1994). Populations with fast response times or better abilities to change growth rates will be able to predominate in mixed populations. It has also been reported previously that bacteria which rapidly formed colonies (a reflection of the short lag-time phase and the growth rate) on nutritionally complex media exhibited higher rRNA operon copy numbers (an average of 5.5 copies), whereas bacteria which responded slowly possessed low rRNA operon copy numbers (an average of 1.4 copies) (Klappenbach et al. 2000). Both representative isolates from the *beta-* and *gamma-Proteobacteria* which were used for the growth experiment are closely related to the genus *Pseudomonas*. They grew fastest in our experiments (Fig. 7). This genus is known to have several species with a high rRNA operon copy number, i.e. *P. putida* (6 copies), *P. stutzeri* (4 copies), *P. fluorescens* (5 copies), *P. balearica (4 copies), P. aeruginose* (4 copies), *P. syringae* (5 copies), *P. aeruginose* (4 copies) (Klappenbach et al. 2000; http://rrndb.cme.msu.edu).

Our isolates belonging to the *alpha-Proteobacteria* group and the *FCB* group grew slower (Fig. 7) compared to the isolates belonging to the *beta-* and *gamma-Proteobacteria.* This might be due to the lower copy number of rRNA operons. Strain *Sphingomonas alaskensis* RB2256, which belongs to the *alpha-Proteobacteria* group for example, possesses only 1 copy of the rRNA operon, whereas the

FCB group possesses only 1 to 2 copies of the rRNA operon (Klappenbach et al. 2000; http://rrndb.cme.msu.edu). Bacteria with higher rRNA operon copy numbers apparently showed higher flexibility and competitiveness values to environmental fluctuations, including temperature and nutrient (Condon et al. 1995) and therefore influenced their abundance in the habitats (Klappenbach et al. 2000).

Methods for determining new species from numerous habitats is currently being discussed vividly (Stackebrandt and Goebel 1994; Roselló-Mora and Amann 2001), since classification based on genotype identification methods is the newest and most dynamic among the different classification approaches for prokaryotic organisms. Species determination techniques have been developed more than two decades ago and were constantly improved by rapid advances in laboratory protocols (Roselló-Mora and Amann 2001). It has been observed that organisms with genomic similarities of more than 70% usually share more than 97% 16S-rRNA sequence similarity. Therefore, new species can be predicted when they exhibit 16S rRNA (gene) sequence identity of less than 97% with other species (Stackebrandt and Goebel 1994). Based on this boundary value, isolate J07, which showed 96.7% similarity with *Clavibacter michiganense* strain JCM 1369 represents a novel species. Most of the isolates in our collection are closely related to known species as depicted from the shallow branch lengths in the phylogenetic tree.

Previous studies reported that psychrotolerant bacteria, not psychrophilic ones, were the predominant ecotypes in permanently cold habitats (DeLille and Perret 1989; Franzmann 1996; Maruyama et al. 1997). Based on their temperature preference, the psychrophilic and mesophilic bacteria appear to be more closely related, than the thermophilic ones (Bowman et al. 1997). Screening for psychrophilic organisms and distinguishing them from psychrotolerant ones is still problematic due to non-specific cultivation methods which would allow to distinguish them (Karl 1993). Improvement in the screening techniques, specifically for psychrophilic bacteria from the many and wide spread low-temperature habitats remains a challenge.

Our isolates showed characteristics of psychrotolerant bacteria. They are capable of growing at higher temperatures when obligate psychrophiles normally would not grow. This is also clearly depicted in the phylogenetic tree, in which most of the closest neighbors of our psychrotolerant isolates branch with non-psychrophilic bacteria. None of our isolates possesses phenotypic features which would allow them to closely cluster with *Curtobacterium (Cryobacterium) psychrophilum* which is a known obligate psychrophilic bacterium.

The results of the growth experiments demonstrate the flexibility of the isolates to grow at different temperatures and nutrient concentrations by adjusting their maximum growth rates and grow even at low temperatures and low nutrient concentrations. This observation suggests that the isolates are well adapted to cope with seasonal temperature variations and with the fluctuation between oligotrophic and mesotrophic conditions. Cells that grow at low nutrient concentrations at low temperatures can quickly respond to temperature changes and nutrient bursts, and, unlike *E. coli*, they

can still grow with appreciable growth rates if temperature and nutrient supply decrease. Lowering the temperature decreases the overal kinetics of enzymatic reactions and low nutrient concentrations favours organisms with high affinity uptake systems (Nedwell and Rutter 1994). Several factors make psychrotolerant oligo- to mesotrophs best suited as members of the high altitude aquatic ecosystems: the ability to survive extremes at low temperatures and food supply, the ability to react quickly once the conditions become more favourable and to regulate growth within a broad temperature and nutrient concentration range. The observed optimum growth temperatures of our isolates were ≥20ºC despite the fact that the in situ temperatures never exceeded 15ºC and enrichment and isolation was carried out at 3°C. Similar growth characteristics were reported previously for microorganisms from low-temperature marine habitats (Isaksen and Jørgensen 1996, Eguchi et al. 1996).

The growth rate values obtained with the mountain lake psychrotolerant heterotrophic bacteria are comparable to the ones reported in a previous study (Isaksen and Jørgensen 1996). Their marine psychrotolerant sulphate reducing bacterium *Desulfurophalus* sp. strain ltk10 grew best at T_{opt} of 18 to 19ºC (μ_{max} 0.030 h^{-1}) while T_{opt} for the mesophilic sulphate reducing bacterium *Desulfobacter curvatus* strain ak30 was at 30 to 35ºC (μ_{max} 0.038 h^{-1}). Nedwell and Rutter (1994) described two Antarctic psychrotolerant isolates, *Hydrogenophaga pseudoflava* (previously *Pseudomonas pseudoflava*) strain 2/10 and *Brevibacterium* sp. strain 1/15, which grew at various glycerol concentrations at temperatures of 2ºC and 16ºC. The growth rates for *H. pseudoflava* in 2 different media containing higher and lower glycerol concentrations were 0.055 h^{-1} and 0.069 h^{-1} at 2ºC, respectively, and 0.139 h^{-1} and 0.0130 h^{-1} at 16ºC, respectively. *Brevibacterium* sp. exhibited the same growth rate values of 0.024 h^{-1} at 2ºC, and 0.095 h^{-1} and 0.098 h^{-1} at 16ºC, respectively.

An isolate of *Sphingomonas* sp. strain RB2256 from an oligotrophic environment showed a very low growth rate of approximately 0.01 h^{-1} at 5ºC, but it was also able to grow at 45ºC at a growth rate which was about 10 times higher (approximately 0.13 h^{-1}). Its optimum temperature was about 37ºC with a μ_{max} of 0.2 h^{-1}. Its maximum growth rate was 0.13 to 0.16 h^{-1} for the entire temperature range and in 10-, 100-, and 1'000- fold diluted complex growth medium (Eguchi et al. 1996). This is in contrast to our isolates which showed remarkably different growth rates in MM (contains 2 mg yeast extract per 1'000 ml medium) and 10 fold diluted LB (contains 500 mg yeast extract per 1'000 ml medium) (Fig. 7). In environments with high nutrient fluctuations it seems essential for microbial populations, to quickly respond by turning protein synthesis up and down in order to make best use of the environmental conditions and to accommodate growth demands (Wagner 1994). Since the ribosomes play a key role in protein synthesis, their rate of formation and their numbers might regulate variations in the growth rate (Wagner 1994), as well as the synthesis of RNAs and proteins (Fegatella and Cavicchioli 2000). These authors observed that RNA and protein synthesis were rapidly increased during the logarithmic growth phase, reached peak rates at the late logarithmic phase, and rapidly decreased during starvation (Fegatella and Cavicchioli 2000).

We found that some isolates were able to express the ability for ice nucleation, a biologically catalyzed transformation of liquid water into solid ice (Gurian-Sherman and Lindow 1993). Microbially mediated ice nucleation represents survival advantage at low temperature condition. A number of investigations describe actively ice-nucleating bacteria as common inhabitants of plant surfaces (Lindow et al. 1978; Lindow et al. 1982), soil (Linderman et al. 1982) or as bacterial symbionts in freeze-tolerant Antarctic beetles (Worland and Block 1999). In our study, the corresponding isolates fall into 2 phylogenetic groups, i.e. the *beta-* and *gamma-* subgroups of the *Proteobacteria*, which are closely related to *Pseudomonas mephitica* ATCC 33665T and *P. fluorescens* IAM12022, respectively. *P. fluorescens* has been reported to belong to the bacteria which are able to catalyze ice formation (Lee et al. 1995), but to our knowledge there was no previous report about the ability of the bacteria belonging to *P. mephitica* in expressing this phenotype.

Bacteria which are able to catalize water crystallization may have an advantage over those which cannot do it. The capable ones will be able to survive frozen environments through slow cellular dehydration. Ice formation on the outside of the cell allows water molecules to move from the cytoplasm across the cell membrane to join crystals of pure water nucleated extracellularly. This increases the osmotic potential inside the cells, thereby preventing freezing and cell damage by ice crystals. The catalized ice nucleation allows for ordered propagation of ice throughout the cell rather than rapid freezing, which can result in membrane rupture and cell death (Baertlein et al. 1992). Since this ability was only found in 6 out of 84 isolates, we must assume that other bacteria use different strategies to survive periods of freezing, for instance by producing anti-freeze proteins (Feller et al. 1996) or other osmolytes.

The genotype and phenotype diversities of culturable heterotrophic bacteria from high mountain lakes increases our appreciations about the richness and heterogenity of the bacterial communities in these extreme habitats. The fact that the phylogenetic affiliation of the isolates fell into only 5 major phylogenetic branches might be due to the limitations in culturing methods and screening processes in which many viable-but-unculturable bacteria were still excluded. Isolates from these habitats showed ecophysiological flexibility in dealing with environmental fluctuations of temperature and nutrient supply by maintaining their population to ensure community survival.

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Environmental fluctuations significantly influence the microbial community composition present in nival lakes

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Abstract:

We studied the temporal (seasonal) and spatial (vertical) distribution of microbial communities in Jöri Lake XIII (2'640 m a.s.l.), an ecologically young alpine lake, and discovered dynamic changes of the community composition related to environmental fluctuations. The iron-rich catchment initiates a unique and interesting mechanism by which the lake ecosystem becomes able to retain the nutrients efficiently.

Temporal Temperature Gradient Gel Electrophoresis (TTGE) of PCR-amplified 16S-rDNA, cloning of the 16S-rDNA from the total community and Fluorescent *In Situ* Hybridization (FISH) were used to monitor the succession of planktic microbial populations. TTGE analysis yielded characteristic seasonal banding patterns. While the lake was stratified the communities at different depths showed distinct banding patterns, whereas during summer upwelling events identical patterns were observed over the entire water column. The TTGE patterns from total community DNA were well represented by libraries of cloned and sequenced 16S-rDNA fragments. Sequencing of 16S-rDNA allowed us to genotypically identify the clones representing predominant bacteria whose DNA appeared in TTGE bands. Out of 45 TTGE bands, 32 were unique whereas 6 out of the remaining 13 were predominant strains present during more than one sampling period. Sequence analysis of 38 TTGE bands showed 88% to 99% similarities to 16S rRNA gene sequences present in databanks. Phylogenetic analysis revealed that the predominant communities belong to *alpha-*, *beta-*, and *delta-*subgroups of *Proteobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Planctomycetes*, and to chloroplasts of a unicellular green alga. FISH and the sequences showed that the highest bacterial diversity stems from the group of the *beta-Proteobacteria*, which also showed the highest seasonal dynamic in Lake Jöri XIII. The archaeal clone libraries were phylogenetically affiliated with both Crenarchaeota and Euryarchaeota lineages originally described from temperate and hyperthermic methane-rich habitats.

1. Introduction

High mountain lake ecosystems have attracted the interests of ecologists mainly because of their extreme physico-chemical conditions, their low nutrient content and their remoteness which makes them least disturbed ecosystems. Recent ecological studies focused on remote, high mountain lake ecosystems because they can be used as sensitive indicators of global warming effects as recorded by climate changes (Psenner and Schmidt 1992). Climate fluctuations indicated by pronounced glacier advances and retreats have significantly influenced alpine aquatic ecosystems (Kamenik et al. 2000).

In this study we analyzed the temporal and spatial distribution of microbial communities of Lake Jöri XIII, which is located in the eastern part of the Swiss Alps. This lake, one among several in this

catchment, was formed after the glacier retreated about 100 years ago and it is no longer under the influence of the glacier today. Lake Jöri XIII is characterized by a low nutrient input but still has an active nutrient cycling. An enormous number of *Chironomidae* tubes found on the bottom of the lake indicate that it has a well-developed, short food web from prokaryotes to insect larvae. Recent studies suggested that its productivity and nutrient cycling is governed by autochthonous processes and by a self trophicating mechanism, rather than by the input from allochthonous sources (Iqbal-Nava, 2003). Most of the lakes in the Jöri catchment are oligotrophic, where the density of planktonic bacteria is naturally regulated by the availability of inorganic and organic nutrients ("bottom-up" control). This population regulation mechanism is stronger than grazing by bacteriovorous flagellates ("top-down" control) (Hinder et al. 1999b).

We studied the microbial community changes in Lake Jöri XIII in relation to the flexibility of the populations in responding to environmental fluctuations. The total community ecotype in these extreme aquatic ecosystems is regularly challenged by seasonal and extreme environmental changes. The habitat is exposed to freezing temperatures, long periods of darkness below an ice and snow cover, and to nutrient deprivation during long winter periods. In summer, it is exposed at the surface to diurnal temperature fluctuations between 0°C and 15°C, to strong light intensities and extremely high UV radiation during ice melt at the height of summer. Due to an active iron cycle the lake is not oligotrophic anymore, as it would be expected from remote mountain lakes far above the tree and vegetation line. The iron cycle has built up mesotrophic nutrient condition, which can lead to organismic blooms in autumn.

In this study, we mainly used culture-independent approaches for the analysis of changes in diversity and predominance. The temporal temperature gradient gel electrophoresis (TTGE), PCR-assisted cloning of the 16S rRNA genes from the total bacterioplankton community and further confirmation by Fluorescence *In-Situ* Hybridization (FISH) techniques were used to study the seasonal and spatial community structure in this lake. Of special interest were microbial community changes during ice melting in summer and during early winter and while the major environmental fluctuation occurred in fall.

Developments in applying molecular biology techniques during the last two decades have revealed enormous microbial diversities, including phylogenetically new branches in the tree of life (Woese et al. 1990). Many studies in microbial ecology are now relying on culture-independent techniques, which are mostly based on the rapid and sensitive polymerase chain reaction (PCR). The PCR technique allows one to retrieve the environmental DNA from the total community, regardless of whether or not they belong to cultivable microorganisms. This method could eliminate the potential biases of the culture-dependent methods, in which only a minority of the microorganisms present can actually be cultivated. In recent years, the techniques for analyzing the microbial diversity within a number of different communities has been further refined and the approach is now widely used in microbial ecology. They include PCR-single-strand conformation polymorphisms (SSCP), temporal

temperature gradient gel electrophoresis (TTGE) and denaturing gradient gel electrophoresis (DGGE). These techniques are PCR-based allowing us to genetically profile the mixed community in various habitats without cultivation (Amann et al. 1995). Furthermore, the diversity, the community composition, the population dynamics, and the species succession can be detected (Øvreas et al. 1997). TTGE and DGGE are basically electrophoretic separations of the PCR products from a mixed population possessing different nucleotide sequences. The linear temperature gradient (in TTGE) or the denaturant concentration gradient (in DGGE) is used to denature the double-stranded DNA during the electrophoresis run (Muyzer et al. 1993; Muyzer and Smalla 1998). Furthermore, a nucleic acid identification method of the sequencing analysis is needed to genetically characterize the community members. The latter can be done by analysing PCR products retrieved from excised TTGE or DGGE bands or from clone libraries corresponding to the TTGE bands (Muyzer et al. 1998).

Another direct and rapid detection technique for the *in-situ* assessment of community structures is FISH. FISH uses specific rRNA-targeted oligonucleotide probes which allow one to taxonomically identify prokaryotes without the need to culture them (Amann et al. 1990; Amann et al. 1995). This method has been widely applied for studying the abundance, the spatial and temporal distribution of the microbial communities in high-mountain lake habitats (e.g. Alfreider et al. 1996; Pernthaler et al. 1998; Glöckner et al. 1999), as well as in marine habitats (e.g. Pernthaler et al. 2002; Pernthaler et al. 2003). The low cellular ribosome content (Binder and Liu 1998), low cell wall permeability (Bidnenko et al. 1998), and inaccessibility of the probe target sites of the rRNA (Fuchs et al. 1998) may limit detection sensitivity in FISH analysis (Amann et al. 1995). However, the availability of helper probes (unlabeled oligonucleotides) can enhance the fluorescence signal in FISH (Fuchs et al. 2000).

2. Materials and methods

2.1. Sampling site and sample collection

Samples were collected from Lake Jöri XIII (2'640 m a.s.l.), one of 21 small lakes in the Jöri catchment area. This area is situated in the eastern Swiss Alps, at 46º46'N latitude and 9º58'E longitude at altitudes between 2'489 m to 3'060 m above sea level. The Lake Jöri XIII has a surface area of approximately 15'400 m^2 and a maximum depth of 10.5 m, it became a seasonally open lake not more than 9 decades ago. This lake has a low sky line towards the east, whereas the west side of the lake is shaded by steep rock walls. This determines the mean global radiation input which is smaller into this lake compared to other lakes in the Jöri catchment (Gabathuler 1999). The bottom of the lake consists of blackened rocks and soft iron oxide containing, organically rich, muddy sediment. The redox iron cycle plays important roles in the bioproductivity of this lake system. The watercolor

of Lake XIII changes from light blue (nearly clear) in early summer, to dark-turbid green in the middle and end of the summer season. This visible water color change indicates the high productivity of the lake; it is due to suspended particles, which are mostly planktic microorganisms.

Samples from the lake water column were collected during different seasons: at ice melting (June 27, 2002), the middle of summer (September 9, 2002), the autumn (October 1, 2002) and early winter when the lake was already totally ice-covered (December 5, 2002). Water samples taken from below the ice on May 31, 2003 were used for making the archaeal clone libraries. Sampling was done with a plastic 500 ml sampler, prerinsed with the lake water from the desired depth. Water samples were taken from 10 depths and the water was filled into sterilized glass bottles. Samples were kept at low temperatures inside a cooling box for transportation.

For DNA extraction, water samples were processed directly in the field laboratory, or immediately after arrival. 75 ml water samples were filtered aseptically through 0.22 μm pore-size filters (Millipore GVWP, diameter 25 mm) using a sterile syringe filtration device. The filters were placed in sterile 1.5 ml tubes and kept at -20°C until further processing. For FISH, 25 to 50 ml water (from the surface, the middle of the water column and near the bottom) were immediately fixed with formaldehyde (2% final concentration, v/v) and incubated at 4°C overnight. Water samples were then filtered through 47 mm diameter bis-phenylene polycarbonate filters (pore-size 0.2 μm, type GTTP; Millipore, Volketswil, Switzerland) and washed with the same amount of sterile water. The filters were stored at -20°C face-up, using sterile petri dishes until further processing for FISH.

2.2. Nucleic acid extraction

For DNA extraction, the filters were placed in sterile 1.5 ml tubes, rinsed with lysis buffer and further processed based on the procedures recommended by the manufacturer (Qiagen Corp., Stanford, USA). Samples were treated applying the protocols for Gram positive bacteria, with the following additional lysis steps. After the cells were enzymatically lysed, 100 mg glass beads (diameter 0.25 mm) were added into the tubes. The tubes were then shaked at 80% maximum speed in a model MM2000 bead-beater (Retsch, Haan, Germany) for 1 min. After bead-beating, samples were further treated according to the protocol described by the manufacturer (Qiagen Corp., Stanford, USA). The tubes containing extracted genomic DNA were stored at -20°C until further processing.

2.3. PCR for cloning and TTGE

The general bacterial primers S-D-Bact-0008-b-S-20 and S-D-Bact-1524-a-A-18 (Table 1) were used to amplify nearly full-lengths of 16S rRNA genes from total community DNA. PCR reactions were done in 25 μl volumes containing a mixture of (final concentrations are given) dH_2O, *Taq* buffer

(1x) (Sigma), 0.1 mg ml^{-1} Bovine Serum Albumin, DNase-free (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTPs, 200 nM of each primer, 40 U ml^{-1} *Taq* Polymerase (Sigma), and approximately 50-100 ng template DNA. PCR was performed with a Techne Thermocycler (Techne LTD, Duxford Cambridge, U.K). PCR was run under the following conditions: initial denaturation at 94ºC for 130 sec. The next steps were 10 cycles of 94ºC for 15 sec, 61ºC for 30 sec and lowering the temperature by 0.5ºC in every cycle, 72ºC for 80 sec. These steps were followed by 20 cycles of 94ºC for 15 sec, 56ºC for 30 sec, 72ºC for 90 sec with increasing the period by 1 sec every cycle followed by a final extension step at 72ºC for 10 min.

Table 1. List of oligonucleotide primers and probes used in this study.

Oligonucleotide	Usage	Sequence (5'- 3')	*E. coli* position	Target	Reference (s)
S-D-Bact-0008-b-S-20	PCR Primer, cloning, sequencing	AGA GTT TGA TCM TGG CTC AG	16S (8-27)	Bacterial domain	Lane, 1991; Hicks et al, 1992
S-D-Bact-1524-a-A-18	PCR Primer, cloning, sequencing	AAG GAG GTG ATC CAR CCG	16S (1524-1541)	Bacterial domain	Lane, 1991
S-D-Bact 341-b-S-17	PCR Primer, TTGE	CCT ACG GGA GGC AGC AG	16S (341-357)	Bacterial domain	Lane, 1991; Muyzer et al, 1998
(GC)-Univ-907-a-A-20	PCR Primer, TTGE	(CGCCCGCCGCGCGCGGCGGGCG GGGCGGGGGCACGGGGGG) CCG TCA ATT CMT TTR AGT TT	16S (907-926)	Bacterial domain	Lane, 1991; Muyzer et al, 1998
Arch-89F	PCR primer, cloning, sequencing	ACG GCT CAG TAA CRC	16S (89 –103)	Archaeal domain	Hershberger et al, 1996
Arch-915R	PCR primer, cloning, sequencing	GTG CTC CCC CGC CAA TTC CT	16S (915 –934)	Archaeal domain	Stahl and Amann, 1991
S-Univ-907-a-A-20	PCR primer for RFLP analysis	CCG TCA ATT CMT TTR AGT TT	16S (907-926)	Bacterial domain	Lane, 1991
S-*-Univ-0519-a-A-18	Intermediate primer for sequencing	GWA TTA CCG CGG CKG CTG	16S (519-536)	Bacterial domain	Lane, 1991
S-*-Univ-0519-a-S-18	Intermediate primer for sequencing	CAG CMG CCG CGG TAA TWC	16S (519-536)	Bacterial domain	Lane, 1991
S-D-Bact-1099-b-S-16	Intermediate primer for sequencing	GYA ACG AGC GCA ACC C	16S (1099-1114)	Bacterial domain	Lane, 1991
S-D-Bact-1099-b-A-16	Intermediate primer for sequencing	GGG TTG CGC TCG TTR C	16S (1099-1114)	Bacterial domain	Lane, 1991
EUBI-III	Probe, FISH	GCT GCC TCC CGT AGG AGT	16S (338-355)	Bacteria, incl. *Verrucomicrobia Planctomycetes*	Daim et al, 1999
NON EUB338	Probe, FISH	ACT CCT ACG GGA GGC AGC	16S (338-355)	Antisense EUB338, Neg. control	Amann et al, 1990
ALF968	Probe, FISH	GGT AAG GTT CTG CGC GTT	16S (968-986)	Alpha-Proteobacteria	Glöckner et al, 1999
BET42a	Probe, FISH	GCC TTC CCA CTT CGT TT	23S (1027-1043)	Beta-Proteobacteria	Manz et al., 1992
GAM42a	Unlabeled competitor of BET42a, FISH	GCC TTC CCA CAT CGT TT	23S (1027-1043)	Gamma-Proteobacteria	Manz et al., 1992
HGC69a	Probe, FISH	TAT AGT TAC CAC CGC CGT	23S (1901-1918)	Actinobacteria	Amann et al, 1995
ARCH915	Probe, FISH	GTG CTC CCC CGC CAA TTC CT	16S (915 –934)	Archaeal domain	Stahl and Amann, 1991
SRB/DSS658	Probe, FISH	TCC ACT TCC CTC TCC CAT	16S (658 –678)	*Desulfosarcina-Desulfococcus*	Manz et al, 1992
M13F	PCR primer to retrieve the clone insert	GTA AAA CGA CGG CCA G	-	Clone inserts in pCR®2.1-TOPO	Invitrogen Corp.
M13R	PCR primer to retrieve the clone insert	CAG GAA ACA GCT ATG AC	-	Clone inserts in pCR®2.1-TOPO	Invitrogen Corp.

PCR products were analyzed by 1% agarose gel electrophoresis in 0.5X TAE running buffer (45 mM Tris-acetate, (pH 8.3) and 4 mM EDTA), to confirm the product size and to estimate the concentration. Gels were stained with ethidium bromide (1 μg ml^{-1}), and photographed under UV. PCR products from representative samples were directly cloned into plasmid vector pCR®2.1-TOPO (3.9 kb), using TOPO TA Cloning Kit and transformed into *Escherichia coli* cells TOP10 (Invitrogen Corp., California, USA).

The general archaeal primers Arch89F and Arch915R were used to amplify the 16S rRNA genes from Archaea. We focused on the archaeal abundance in the bottom part of the lake, since FISH analysis had revealed large numbers of archaea in deep water samples (see results). PCR products obtained with the universal archaeal primers were cloned into *E. coli* cells applying the protocols described above.

For the TTGE analysis, universal bacterial primers S-D-Bact-341-b-S-17 and a GC clamp primer GC-Univ-907-a-A-20 were used to amplify approximately 560 bp of the rRNA genes present in the extracted DNA. 1 μl PCR product contained extracted DNA as template in a nested PCR. This increased the sensitivity of the amplification and the signal. PCR was run under the following conditions: initial denaturation at 94°C for 5 min, followed by 75°C for 15 sec, 20 cycles of 94°C for 20 sec, 65°C -lowered by 0.5°C after each cycle- for 30 sec, and elongation temperature 72°C for 1 min, followed by 15 cycles of 94°C for 20 sec, 52°C for 30 sec, 72°C for 70 sec, and a final extension step at 72°C for 10 min.

2.4. Community profiling from the samples by TTGE

TGGE was carried out with a DCode Mutation Detection system (Bio-Rad Laboratories). 10 μl of the PCR samples and 10 μl of the 2X loading buffer (70% [v/v] glycerol, 0.05% [w/v] bromophenol blue) were loaded onto 6% polyacrylamide gels (acrylamide: N,N'-methylene bis-acrylamide 37.5:1 [w/w]; 7 M urea, 1X TAE). The gels were run at temperatures ranging from 52°C to 70°C, temperature ramping rate was 1.3°C h^{-1}, the voltage was 90V (4.0V/cm), and running time was about 13 h. The gels were stained in 1 μg ml^{-1} ethidium bromide solution for 15 min, destained in water for 45 min, visualized, and photographed under UV transillumination.

2.5. Identification of predominant bands in TTGE

Genotype identification of predominant microbes was performed by sequencing the DNA from PCR-TTGE bands and the clones representing them. All well separated dominant bands from the gels were excised aseptically by a sterile razor blade and placed in sterile 1.5 ml tubes. Each excised band was mixed with 20 μl sterile water and incubated overnight at -20°C and at 4°C for 12 h to allow the

DNA to diffuse out of the gel. 10 to 15 µl DNA template from the supernatant were then used as template for the PCR reamplification. DNA was reamplified with S-D-Bact-341-b-S-17 and GC-Univ-907-a-A-20 to verify the original mixture DNA in the same TTGE gel. In addition, the primer S-D-Bact-341-b-S-17 and the non-GC primer S-Univ-907-a-A-20 were also used to prepare an RFLP analysis of these bands. RFLP analysis was done using *Hae*III and *Hinf*I restriction endonuclease and run in polyacrylamide gels for 30 to 60 min.

Clones representing the TTGE bands were selected from the libraries. All inserts in the clone libraries were characterized based on their RFLP patterns and TTGE migration on the gels. Initially, all white colonies were PCR-amplified employing the M13F and M13R primer set. The PCR products were then subjected to PCR reamplifications either with S-D-Bact-341-b-S-17 and GC-Univ-907-a-A-20 (for TTGE analysis) or by the primer S-D-Bact-341-b-S-17 and the non-GC primer S-Univ-907-a-A-20 (for RFLP analysis). Furthermore, the clones representing the dominant DNA bands on TTGE gels can be determined by comparing their migration on TTGE gels, their RFLP patterns with the excised bands from the TTGE gels, and finally by checking the clone candidates by comparing with the environmental samples in the same TTGE gel.

2.6. Sequencing of the 16S rRNA genes from PCR-TTGE bands and the clones

The DNA fragments of the dominant bacteria which appeared in TTGE gels were sequenced for genotype identification. PCR products of both DNA strands of the bands were sequenced with S-D-Bact-341-b-S-17 and the non-GC primer S-Univ-907-a-A-20. PCR products of the clones (amplified by M13F and M13R primers) were purified employing microcon centrifugal filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA). Almost full-lengths of the 16S rRNA gene were bidirectionally sequenced using ABI Prism® Big Dye™ v2.0 (Applied Biosystems). The following primers were used for the PCR reactions, i.e. S-D-bact-0008-b-S-20, S-*-Univ-0519-a-A-18, S-*-Univ-0519-a-S-18, S-D-Bact-1099-b-S-16, S-D-Bact-1099-b-A-16, and S-D-bact-1524-a-A-18 (Table 1). For a 10 µl-single PCR reaction, 5 to 20 ng DNA template, 3 µl Big Dye v.2 (Applied Biosystems) and 3 µl of 1.5 µM primer were used. After the sequencing PCR, the products were purified with Sephadex G-50 (Amersham, Pharmacia Biotech AB) and loaded onto the sequencer (Applied Biosystems, ABI Prism 3100 Genetic Analyzer). The rRNA gene sequences were submitted to GenBank and have been assigned accession numbers AJ867849 to AJ867893 (for the PCR-TTGE bands) and AJ867894 to AJ867932 (for the clones).

2.7. Identification of archaea present in Lake Jöri XIII

The archaeal clone libraries which emerged from amplifying with the general archaeal primers Arch-89F and Arch-915R (Table 1) were checked with RFLP analysis. Whole cell PCR employing the M13F/M13R primer set was applied to all 104 white colonies in order to retrieve the fragments of archaeal 16S rRNA genes from the clones. RFLP analysis was done with *Hae*III and *Hinf*I restriction endonuclease and run in 6% polyacrylamide gels for 1 h. Polyacrylamide gels were run in a DCode Mutation Detection system (Bio-Rad Laboratories) without applying different temperature ramping rates. Clones representing different RFLP patterns were selected for further sequencing analysis.

The PCR products of the unique archaeal clones (amplified with the M13F/M13R primer set) were purified through the microcon centrifugal filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA). The fragments of 16S rRNA genes were bidirectionally sequenced using ABI Prism® Big Dye™ v2.0 (Applied Biosystems). The primers used for the PCR reactions were Arch-89F and Arch-915R. The sequencing PCR procedure followed the steps outlined above.

2.8. Phylogenetic analysis

The closest neighbors of the sequences were searched with the aid of the BLAST Search tool program available from NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). The 16S rRNA gene sequences of the clones representing dominant TTGE bands and their closest neighbor sequences obtained from the BLAST program (if not already in the ARB databank) were imported into the ARB software environment (http://www.arb-home.de/). The new sequences were imported to the ARB database (release May 2002) and aligned automatically employing the Fast Aligner V1.03 of the ARB phylogeny software package. The alignment was subsequently corrected manually based on secondary structure information and based on the sequences of the closest relatives as references by using the maximum likelihood method available in the ARB package. The sequences were added to the consensus tree in the ARB database, and the distances were calculated by using the maximum-parsimony approach. The overall phylogenetic affinity was evaluated using a consensus filter of *Escherichia coli*. The phylogenetic trees were constructed by the neighbor-joining method as provided in the ARB program. Bootstrap analyses based on 100 resamplings were performed in order to test the robustness of the inferred topologies in the phylogenetic trees.

2.9. Fluorescent In-Situ Hybridization (FISH)

Filters containing concentrated cells were thawed and cut into sections. Filter sections were hybridized with 150 ng of Cy3-labeled probes. A bacterial domain probe of EUBI-III was used, and a

non-specific binding probe of NON EUB338 served as the negative control. The following group-specific probes were used: ALF968 (*alpha-Proteobacteria*), BET42a (*beta-Proteobacteria*) was used together with its unlabeled-competitor of GAM42a, HGC69a (*Gram positive, High G+C* bacteria), DSS/SRB658 (Sulfate-reducing bacteria, *Desulfosarcina-Desulfococcus* group), and an archaeal domain probe ARCH915 (all sequences and target positions of the probes and competitor probes are listed in Table 1). FISH of filter sections with oligonucleotide probes, were counterstained with DAPI (4', 6'-diamino-2-phenylindole). They were then mounted as previously described (Glöckner et al. 1996). Cells were viewed under a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany). For each group-specific probe, stained-cells were counted among 500 to 1'000 DAPI-stained cells.

3. Results

3.1. Physical and chemical parameters in Lake Jöri XIII

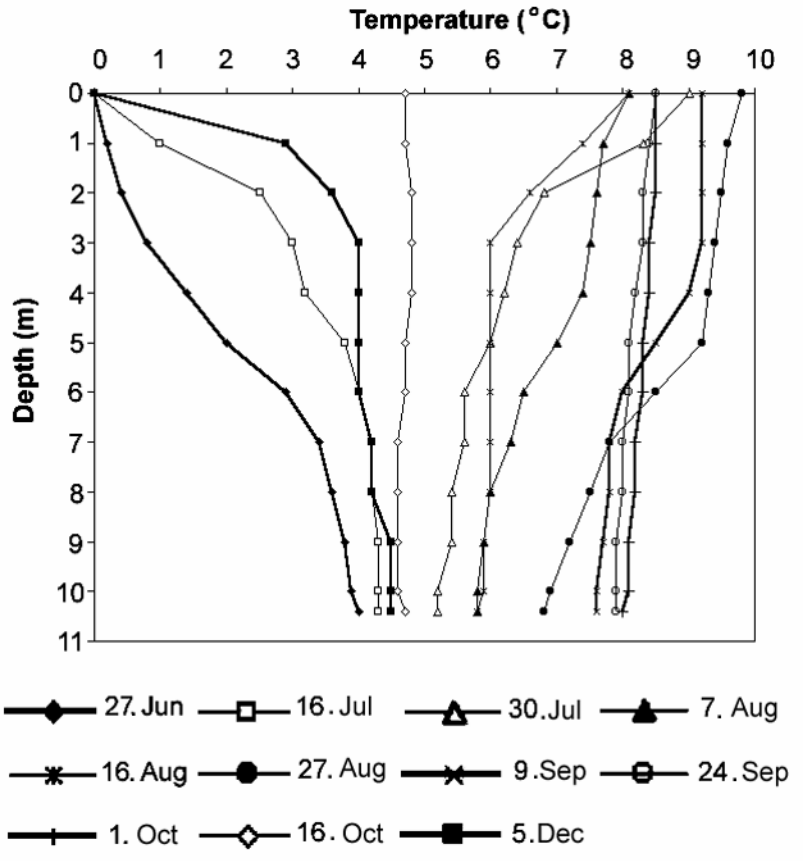

Figure 1. Temperature profiles of Lake Jöri XIII in 2002. Temperature profiles shown as thick lines are for the dates when samples for the total community analysis were collected.

Seasonal temperature changes for 2002 in Lake Jöri XIII are shown in Fig. 1. Thermally stratified conditions prevailed below the ice, which normally lasts 7 to 8 months, whereas destabilization can occur several times during the ice-free season (Fig. 1). The ice melting period normally starts between the middle and the end of July; at the beginning of August, the ice cover has normally completely melted. The surface temperature increases quickly and the lake reaches a short period of thermal stratification (30 July until 27 August). The lake has a maximum depth of 10.4 m, which is too shallow to maintain a stable summer stratification for extended periods of time. The temperature of the water column can become homothermic and the water masses can mix again as a consequence of snow fall and atmospheric temperature drops, even during the height of summer.

Strong winds can also influence summer thermal stratification, as recorded on 9 September, 24 September, 1 October and 16 October. During these representative dates, the temperature profiles indicate mixing events of the entire water column. The maximum temperatures of the lake, which seldom exceeded 12°C in 2002, were measured in September and early October. Early winter sampling was done on 5 December when the lake was totally ice-covered.

The chemical parameters of Lake Jöri XIII are compared with those from other high mountain lake ecosystems (Table 2). The chloride and nitrate values are low which indicates that these habitats are not heavily influenced by anthropogenic activities. Sulphate concentration is low, since the catchment does not contain gypsum or anhydrite rocks. High ammonium values are caused by ammonium release from the sediment and mixing. The scavenging system which is governed by the iron chemistry in this iron-rich habitat is able to retain the phosphate in the lake. Therefore, its concentrations (including total and soluble reactive phosphorous) are higher than those measured in high mountain lakes which are not located in iron-rich catchments.

Table 2. Chemical conditions in selected high-mountain Alpine lakes compared with Lake Jöri XIII.

Lake, Country, Year	Chloride [µM]	Nitrate [µM]	Sulphate [µM]	Ammonium [µM]	TP, SRP [µM]	Conductivity *) [µS/cm]	Reference
Jöri Lake XIII, Switzerland 2002	2	4	21	1.7	0.41	6 - 25	Iqbal-Nava, 2003
Jöri Glacier, Switzerland 2002	nd	nd	nd	nd	< 0.05	nd	Iqbal-Nava, 2003
Jöri Lake III, Switzerland 1996	5	14	31	0.6	1.2	20	Hinder, 1999a
Jöri Lake III, Switzerland 1997	2	18	26	0.6	0.3	16	Hinder, 1999a
Jöri Lake VII, Switzerland 1996	6	13	21	nd	nd	18	Hinder, 1999a
Jöri Lake VII, Switzerland 1997	nd	12	15	0.2	0.3	nd	Hinder, 1999a
Lake Gossenkölle, Austria 1999	3	19	21	0.3	0.003-0.07	21	Kamenik, 2000
Lake Schwarz, Austria 1993/94	5-7	11-13	35-43	0.6-1.8	0.1	13-16	AL: PE
Lake Paione Superiore, Italy 1999	nd	25	2.6	0.1	0.05	9	AL: PE

nd: not determined, SRP: soluble reactive phosphorous, *) normalized to 25ºC.

Table 3 shows the average values of soluble phosphate and dissolved iron of Lake Jöri XIII in 2002. In the course of the year two events with peak concentrations of soluble phosphate ranging from

0.07 to 0.92 µM occurred on the lake bottom. The iron concentrations were high at the lake bottom under anoxic conditions in July and again after the ice cover had formed in December. Above 10 m depth the soluble iron concentration is small as ferrous iron is oxidized under oxic conditions, and it precipitates and sediments again.

Table 3. Soluble phosphate and dissolved iron values of Lake Jöri XIII in 2002*).

Date		2 Jul 02	9 Jul 02	16 Jul 02	30 Jul 02	14 Aug 02	9 Sep 02	21 Sep 02	30 Sep 02	16 Oct 02	5 Dec 02
Soluble phosphate (µM)	ξ (n)	0.20 (6)	0.07 (7)	0.05 (4)	0.09 (2)	0.46 (7)	0.84 (7)	0.70 (5)	0.68 (10)	0.23 (10)	0.13 (9)
	SD	0.15	0.07	0.01	0	0.03	0.05	0.06	0.14	0.13	0.05
Dissolved iron (µM)	ξ (n)	nd	40.0 (3) **)	1.75 (4) **)	2.62 (5) **)	11.47 (3) **)	0.0 (1)	0.5 (2)	0.2 (1)	0.0 (1)	1.65 (4) **)
	SD	nd	43.17	2.69	3.72	15.93	0	0.05	0	0	1.96

*) Data from Iqbal-Nava (2003), ξ: mean, n: number of samples, SD: standard deviation, nd: not determined.
**) Dissolved iron from the water column near sediment of the lake showed higher values compared to the samples from the water column, range 5 to 100 µM.

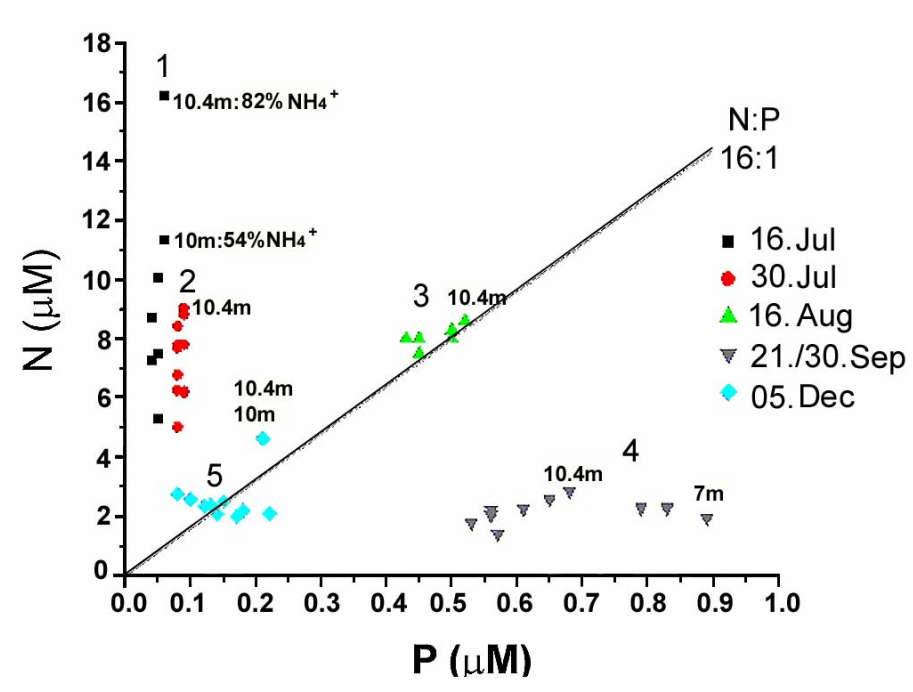

Figure 2. Changing nutrient status of Jöri Lake XIII. P (dissolved inorganic and organically bound phosphate) plotted against N ($NH_4^+ + NO_3^-$). The straight line indicates the Redfield ratio. The depth for extremes is given in meters. Numbers 1 - 5 refer to the description of events in the text.

The trophic status of Lake Jöri XIII can be illustrated by comparing nitrogen and phosphorous values (Fig. 2). These nutrient compounds often become the limiting factors in aquatic ecosystems. The Redfield ratio, the stoichiometric ratio of C:N:P (106C: 16N: 1P) found in planktonic biomass grown without N or P limitation (Falkowski, 2000), can be used to describe changes in the trophic state of the lake ecosystem and to understand seasonal productivity variations. The data points which fall onto the straight line (16N : 1P) signify balanced N and P conditions for algal growth. The changes in trophic conditions between P-limitation in spring and N-limitation in fall are almost entirely regulated by microbially mediated processes.

The natural changes in the trophic states of this lake can be understood as follows. Before the ice melts in July, the water at the bottom is anoxic as indicated by the high concentrations of the NH_4^+, Fe (II), and SRP. In early summer (30 July), after the ice has melted, the water masses were mixing which brought oxygen-rich-water to the ground. Aerobic organisms could now consume the NH_4^+ in the entire water column. The lake mixing events in July led to an increase of the phosphate concentration in the entire water column while the ferrous iron got immediately reoxidized at the sediment water interface. Due to the higher water temperatures in August, microbial growth increased. The growing conditions for phototrophic microorganisms were now optimal and nitrogen becomes limiting while the phosphate concentration reaches its maximum. At the beginning of winter (5 December), the N:P ratio was balanced but at very low concentrations for both elements.

3.2. Microbial community structure in Lake Jöri XIII

To study the spatial and seasonal microbial population shifts in the Lake Jöri XIII, the community composition in all samples from various depths and seasons was compared. The samples from each sampling date gave 10 to 12 discrete bands in TGGE gels. The bands correspond to the most abundant bacterial groups, and different band patterns reflect distinct microbial populations in their environment (Muyzer et al. 1993). The number of bands may not accurately represent all microbial populations present in the lake, but the number of predominant populations in the microbial community in situ (Muyzer et al. 1993, Muyzer and Smalla 1998). Microbial populations which did not appear in the TTGE pattern are those present in low abundances at the particular sampling date.

Water column mixing significantly influences the vertical distribution of the planktonic microbial populations. TTGE patterns of samples taken while the lake was stratified yielded distinct banding patterns for different depths and seasons whereas identical banding patterns over the entire water depth were observed during downwelling and upwelling events (Fig. 3).

TTGE showed different banding patterns, indicating the occurrence of microbial population shifts over the seasons. Some bands from the samples taken from different ice cover periods, appeared inconstantly in the water column. Band A11 appeared at depths from just below the ice cover until the middle of the water column, but it was not present in the lower layers of the water column. Several

bands just appeared in the lower water column i.e. band A1 (appeared at depths from 3 to 8.8 m), band A2 (at depths 5, 7 and 8.8 m) and band A3 (at depths between 7 and 8.8 m): These bands were completely absent in samples taken from near the surface. Several other bands (A4, A5, A6, A7, A8, A9 and A10) occurred with different intensities at all depths. The TTGE profiles obtained for the early winter sampling of December 5 again showed a distinct microbial population. Bands D1, D9 and D10 only occurred in the lower part of the water column, band D8 only existed between the surface and the middle of the water column, whereas bands D11 and D12 clearly appeared just below the ice cover and down to depths of 4 m to 5 m.

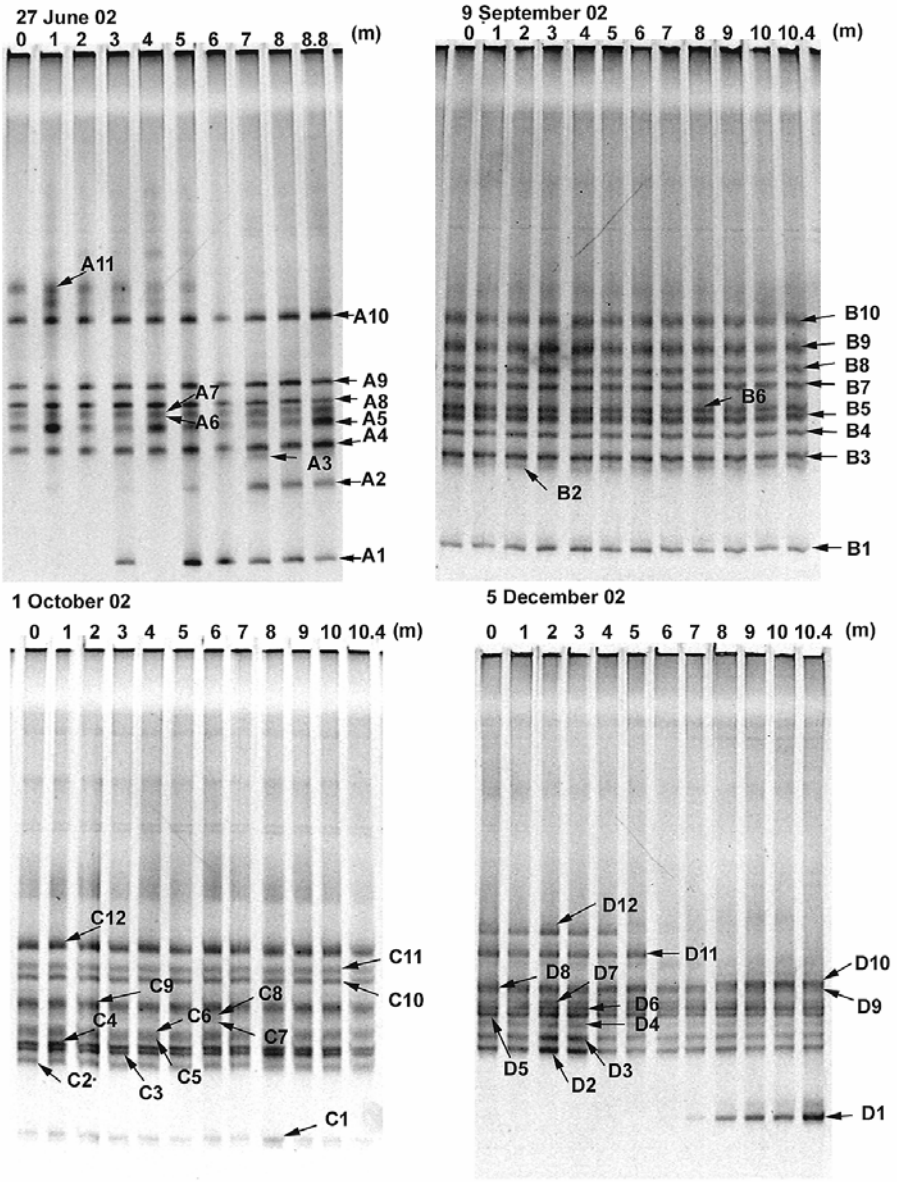

Figure 3. TGGE patterns of total community DNA from Lake Jöri XIII collected at different depths for 4 sampling dates over the year. Numbered letters and arrows indicate excised bands for RFLP and sequenced clones. See also Table 4.

Samples taken from various depths at two different sampling dates during the summer season did not reveal distinct TTGE banding patterns. Bands B1 to B10 appeared in all samples taken on 9 September more or less at the same intensity. TTGE patterns from samples of 1 October showed that bands C1 to C12 appeared at various depths. Bands C9 and C12 gave a stronger signal compared to other bands. The similarity of the TTGE banding patterns may also be used as test for the reproducibility of the DNA extraction method, the PCR amplification, and the TGGE analysis. The similarity of TTGE pattern in the entire water column may indicate the presence of similar bacterial populations at all depths.

To monitor the seasonal and spatial community shifts, we constructed the clone libraries and compared them to the corresponding TTGE bands. This allowed us to identify the most predominant populations and to detect their appearance and disappearance as the conditions changed. Bacterial clone libraries were constructed from samples of the upper layer and from the bottom layer of the water column while the lake was thermally stratified (27 June and 5 December). Representative clone libraries for summer conditions originated from the total community DNA of the middle of the water column. Altogether, 225 clones from the clone libraries were analyzed.

The majority of the TTGE bands were well represented by sequenced fragments from the clone libraries. Identification of 45 TTGE bands by using RFLP analysis and individual TTGE runs of the clones revealed that 32 bands were unique whereas 6 out of the remaining 13 bands represented predominant strains present in more than one sampling period. Bands which appeared in the winter and the autumn samples were A1 (shares with B1); C1 (shares with D1), A10 (shares with C12); A11 (shares with B10); B9 (shares with C11 and D12), and bands which occurred in summer and autumn samples were B7 (shares with C9). All other bands occurred in one season only.

Cloning of 16S-rRNA genes from total community DNA and sequencing them allowed us to genotypically identify the clones that revealed dominant TTGE bands. The phylogenetic affiliation of the clones representing the most abundant microbial communities is depicted in Fig. 4a and Fig. 4b. Cluster analysis revealed that these bacteria belong to the following phylogenetic groups: the *Proteobacteria* (*alpha-, beta-*, and *delta*-group), the *Actinobacteria*, the *Verrucomicrobia*, the *Planctomycetes*, the OP10, the Cyanobacteria, and to a number of uncultured bacteria. The highest bacterial diversity stems from the group of the *beta-Proteobacteria* which is the most dominant one during all seasons. More than 50% of the clones analysed (i.e. 23 sequences out of 38 different clones) belong to this subgroup. Many nearest neighbors in this subgroup are bacteria or clones previously found in other cold environments, i.e. Arctic Lake Toolik, Arctic sea ice, Antarctic microbial mats, southern hemisphere glaciers, Austrian Central Alps Lake Gossenkölle, and other aquatic habitats i.e. Lake Fuchskuhle, Wesser estuary, Lake Loosdrecht, Wulfbach river, and Elbe river.

The sequence similarity values to the closest neighbors of the most abundant microbial communities in Lake Jöri XIII are summarized in Table 4. Sequence analysis of 38 TTGE bands showed 88 to 99% similarities to 16S rRNA gene sequences present in the databanks. Only 23

sequences out of the 38 almost full length 16S rDNA sequences are more than 97% similar to sequences present in the databank, whereas 15 others showed similarities less than 97%.

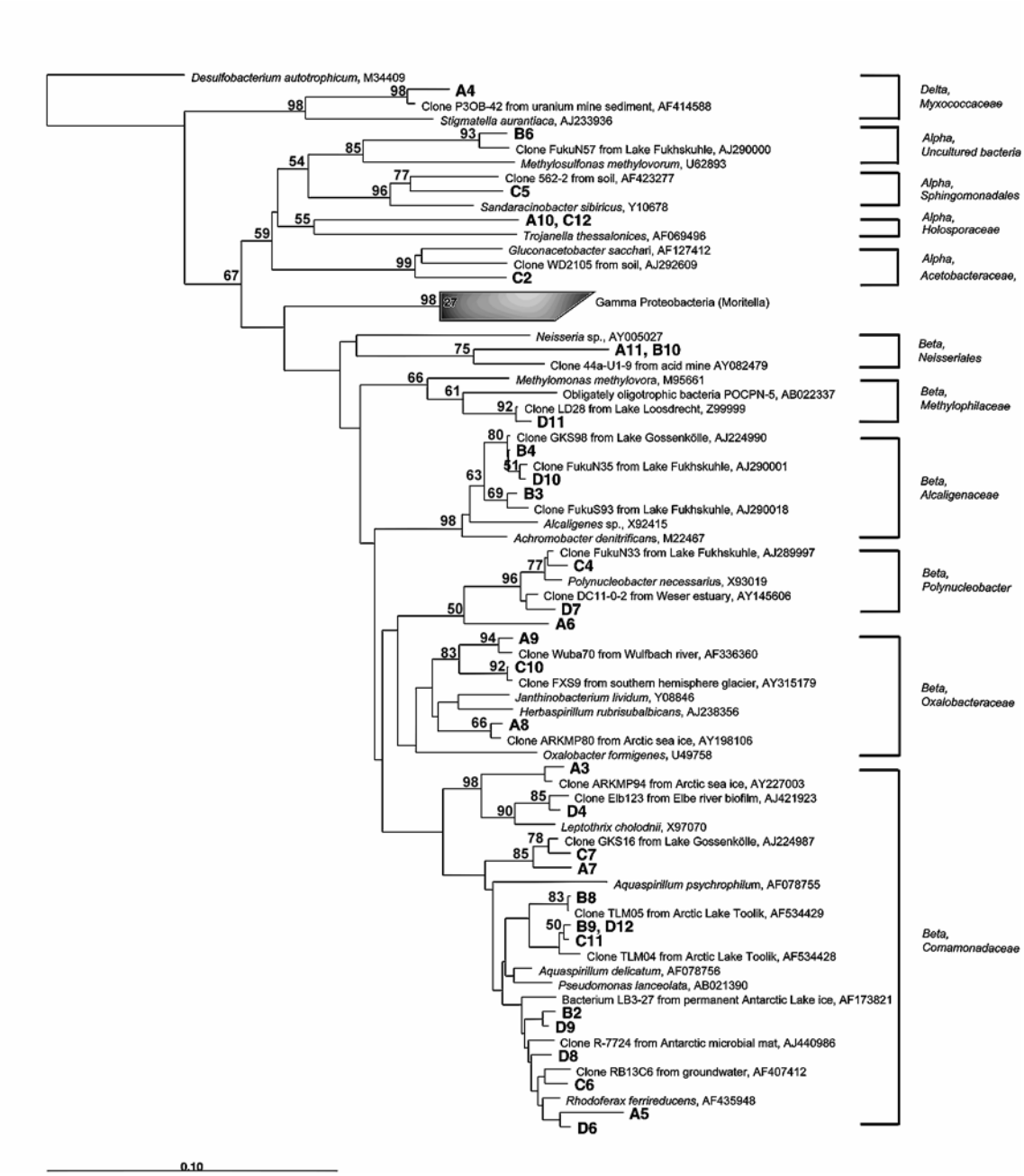

Figure 4a. Phylogenetic affiliation of predominant Proteobacteria. *Desulfobacterium autotrophicum*, M34409 (a sulphate reducer) is used as an outgroup organism. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar indicates 10% sequence divergence.

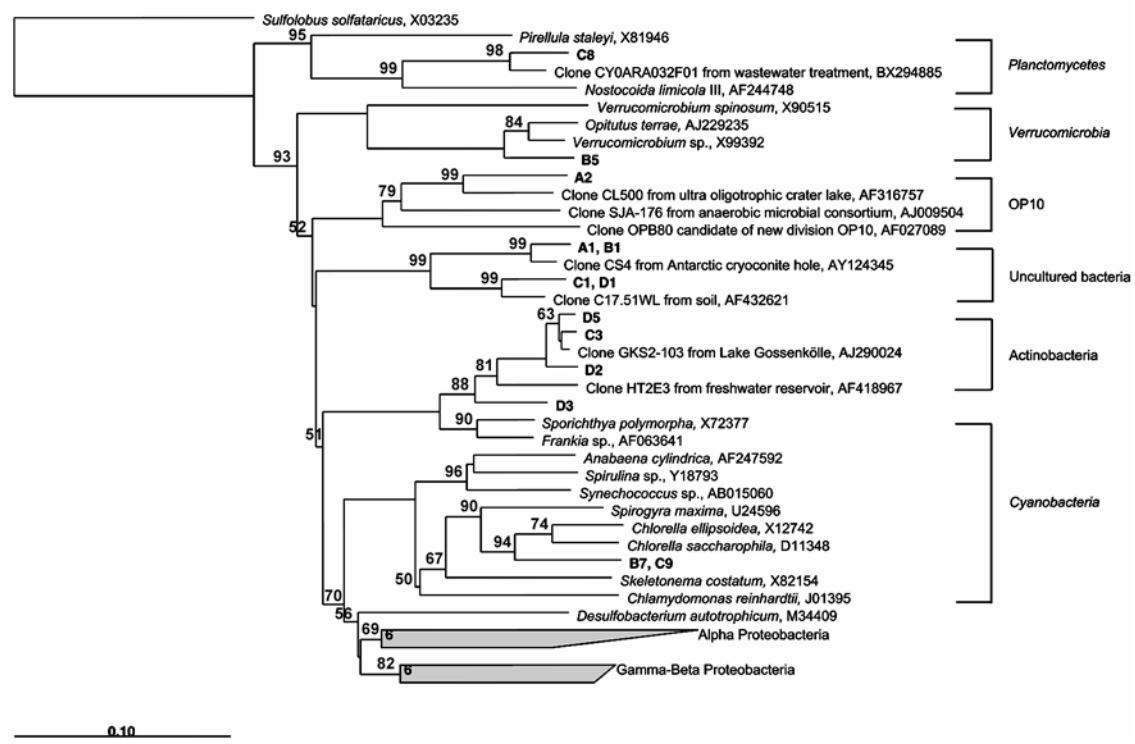

Figure 4b. Phylogenetic affiliation of predominant communities of non-Proteobacteria. A sulphate and sulfur reducing bacterium (*Sulfolobus sulfataricus*, X03235) is used as an outgroup organism. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar indicates 10% sequence divergence.

Most of the novel bacterioplankton from Lake Jöri XIII reveals sequences which are related to clones found in various geographical locations. They show sequence differences of 3 to 12%. Their closest known relatives with similarity values between 86 to 97% were the following (GenBank descriptions, unless otherwise noted): A1 (shares with B1) and C1 (shares with D1) are related to *Gemmatimonas aurantiaca,* an aerobic, Gram-negative, polyphosphate-accumulating bacterium, A2 is distantly related (only 87% sequence similarity) to a spore-forming, sulphate-reducing mesophilic bacterium *Desulfosporosinus meridiei* (GenBank accession no. AF076246). B5 is distantly related to the *Verrucomicrobiae* pACH90 (95% sequence similarity), B7 (shares with C9) is related to the chloroplast of the unicellular green alga, *Chlorella saccharophila* strain 3.80, C8 is related to *Nostocoida limicola* III strain Ben220 (member of the *Planctomycetes*) which is often found in wastewater treatment plants. D2 related to *Frankia* sp. strain Dryas (*Actinobacteria*) is a nitrogen-fixing symbiont which belongs to the *Actinomycetales*. D3, distantly related to *Sporichthya polymorpha* (92% sequence similarity), a facultative anaerobe and grouped with the Gram-variable-type cells. The young cells tend to be Gram negative, older cells have a Gram-positive type cell wall.

The new sequences which are affiliated to the *Proteobacteria* are A5, and bands of C6 and D6 stem from organisms which are related to a psychrotolerant, facultative anaerobic bacterium that oxidizes acetate coupled to Fe (III) reduction: *Rhodoferax ferrireducens* (GenBank accession no.

Table 4. Identification of Lake Jöri XIII clones corresponding to the dominant TTGE bands in Fig. 3.

Clone (s) designation	Accesio n no	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest–described isolate, accession no.	% Similarity
A1-B1	AJ867894	1476	Gemmatimonadetes	Clone CS4, AY124345	Antarctic cryoconite hole	97	*Gemmatimonas aurantiaca*, AB072735	97
A2	AJ867895	1465	Unclassified bacteria	Clone Crater Lake CL500-48, AF316757	Ultra oligotrophic Crater Lake	92	*Desulfosporosinus meridiei* S4, AF076246	87
A3	AJ867896	1486	Beta-Proteobacteria	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Matsuebacter chitosanotabidus*, AB006851	96
A4	AJ867897	1491	Unclassified bacteria	Clone P3OB-42, AF414588	Uranium mine sediment	99	*Stigmatella aurantiaca* strain Sg a15, SAU233936	91
A5	AJ867898	1171	Beta-Proteobacteria	*Rhodoferax ferrireducens*, AF435948	Strain *Rhodoferax ferrireducens*	97		
A6	AJ867899	1483	Beta-Proteobacteria	Clone DC11-0-2, AY145606	Weser estuary	95	*Ralstonia campinensis*, AY040358	93
A7	AJ867900	1487	Beta-Proteobacteria	Clone RA13C6, AF407405	Monochlorobenzene contaminated groundwater	98	*Polaromonas naphthalenivorans*, AY166684	97
A8	AJ867901	1483	Beta-Proteobacteria	Clone ARKMP80, AY198106	Arctic sea ice	99	*Ultramicrobacterium* str. ND5, AB008506	97
A9	AJ867902	1489	Beta-Proteobacteria	Clone Wuba70, AF336360	Wulfbach river	99	*Herbaspirillum lusitanum*, AF543312	95
A10-C12	AJ867903	1455	Alpha-Proteobacteria	Bacterium Ellin328, AF498710	Soil isolate Ellin 328	88	*Trojanella thessalonices* AF069496	89
A11-B10	AJ867904	1499	Beta-Proteobacteria	Clone 44a-U1-9, AY082479	Subsurface acid mine drainage system	93	*Simonsiella steedae* strain ATCC29457, AF328156	88
B2	AJ867905	1527	Beta-Proteobacteria	Clone SJA-62, AJ009470	Microbial consortium	97	*Aquaspirillum delicatum*, AF078756	97
B3	AJ867906	1523	Beta-Proteobacteria	Clone FukuS93, AJ290018	Lake Fuchskuhle	98	*Achromobacter xylosoxidans*, AJ278451	96
B4	AJ867907	1521	Beta-Proteobacteria	Clone GKS98, AJ224990	Lake Gossenkölle	99	*Kinetoplastibacterium crithidii*, L29303	94
B5	AJ867908	1552	Verrucomicrobia	*Verrucomicrobiae* pACH90, AY297806	Waterlogged archaeological wood	95		
B6	AJ867909	1477	Alpha-Proteobacteria	Clone FukuN57, AJ290000	Lake Fuchskuhle	98	*Bosea minatitlanensis*, AF273081	94
B7-C9	AJ867910	1695	Unicellular green alga, chloroplast	*Chlorella saccharophila* (strain 3.80), D11348	*Chlorella saccharophila*	96		
B8	AJ867911	1523	Beta-Proteobacteria	Clone TLM05/ TLMdgge10, AF534429	Lake Toolik, Arctic	99	*Aquaspirillum delicatum*, AF078756	97
B9-D12	AJ867912	1482	Beta-Proteobacteria	Clone TLM04/ TLMdgge03, AF534428	Lake Toolik, Arctic	99	*Aquaspirillum delicatum*, AF078756	97

Table 4. Continue

Clone (s) designation	Accession no	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest–described isolate, accession no.	% Similarity
C1-D1	AJ867913	1477	Unknown	Clone C17.51WL, AF432621	Pine rhizosphere soil	96	*Gemmatimonas aurantiaca*, AB072735	90
C2	AJ867914	1444	Unknown	Clone WD2105, AJ292609	Soil	95	*Gluconacetobacter sacchari*, AF127412	94
C3	AJ867915	1463	Actinobacteria	Clone GKS2-103, AJ290024	Lake Gossenkölle	99	*Frankia* sp. strain FE37, AF063641	91
C4	AJ867916	1476	Beta-Proteobacteria	Clone FukuN33, AJ289997	Lake Fuchskuhle	99	*Polynucleobacter necessarius*, X93019	98
C5	AJ867917	1439	Alpha-Proteobacteria	Clone 565-2, AF423277	Soil	93	*Sphingomonas* sp. strain eh2, AF548567	92
C6	AJ867918	1487	Beta-Proteobacteria	Clone RB13C6, AF407412	Monochlorobenzene contaminated groundwater	98	*Rhodoferax ferrireducens*, AF435948	98
C7	AJ867919	1478	Beta-Proteobacteria	Clone GKS16, AJ224987	Lake Gossenkölle	99	*Polaromonas vacuolata*, U14585	97
C8	AJ867920	1402	Planktomycetes	Clone CY0ARA032F01, BX294885	Wastewater treatment plant	96	*Nostocoida limicola* III strain Ben220, AF244748	86
C10	AJ867921	1487	Beta-Proteobacteria	Clone FXS9, AY315179	Southern hemisphere glaciers	99	*Ultramicrobacterium* strain MY14, AB008503	95
C11	AJ867922	1482	Beta-Proteobacteria	Clone TLM04/ TLMdgge03, AF534428	Lake Toolik, Arctic	99	*Aquaspirillum delicatum*, AF078756	97
D2	AJ867923	1463	Actinobacteria	Clone FukuN30, AJ289996	Lake Fuchskuhle	97	*Frankia* sp. strain Dryas, L40616	91
D3	AJ867924	1466	Unclassified bacteria	Clone HT2E3, AF418967	Freshwater reservoir	93	*Sporichthya polymorpha*, AB025317	92
D4	AJ867925	1482	Beta-Proteobacteria	Clone Elb123, AJ421923	Biofilms of Polluted Rivers, Elbe River	98	*Leptothrix cholodnii* strain CCM 1827, X97070	97
D5	AJ867926	1465	Actinobacteria	Clone FukuN30, AJ289996	Lake Fuchskuhle	99	*Frankia* sp. strain FE37, AF063641	91
D6	AJ867927	1484	Beta-Proteobacteria	Clone GOUTB7, AY050594	Monochlorobenzene contaminated groundwater	98	*Rhodoferax ferrireducens*, AF435948	98
D7	AJ867928	1480	Beta-Proteobacteria	Clone MWH-MoIso1, AJ550671	Freshwater bacterioplankton	98	*Polynucleobacter necessarius*, X93019	97
D8	AJ867929	1487	Beta-Proteobacteria	Clone H-5, AF523038	Natural mineral water	99	*Aquaspirillum delicatum*, AF078756	97
D9	AJ867930	1483	Beta-Proteobacteria	Isolate R-7724, AJ440986	microbial mat, Antarctic lakes	98	*Aquaspirillum delicatum*, AF078756	97
D10	AJ867931	1479	Beta-Proteobacteria	Clone GKS98, AJ224990	Lake Gossenkölle	99	*Bordetella avium* ATCC 35086, AF177666	97
D11	AJ867932	1490	Beta-Proteobacteria	Clone LD28, Z99999	Lake Loosdrecht	99	*Aminomonas aminovorus* strain C2A1; AY027801	94

AF435948). A6 is distantly related to Ralstonia campinensis (GenBank accession no. AY040358, 93% sequence similarity) which is a metal-resistant bacterium originally isolated from industrial biotopes. A10 (shares with C12) is distantly related (89% sequence similarity) to *Trojanella thessalonices*, a bacterium previously found as obligate endosymbiont and intracellular parasite of Acanthamoeba species. A11 (shares with B10) is related to *Simonsiella steedae* strain ATCC29457 (GenBank accession no. AF328156). Its population predominated at the end of the ice cover period in June and its abundance in the surface water layer was higher than in lower water layers (Fig. 3, TTGE pattern, 27 June).

Sequences of the clones represented by TTGE bands B2 and B8, B9, C11, D12, D8 as well as D9 are related to *Aquaspirillum delicatum,* AF078756 with 97% sequence similarity. C2 is distantly related to *Gluconacetobacter sacchari* isolate IF2-6 (GenBank accession no. AF127412, 94% sequence similarity), C5 is distantly related to a novel endolithic community found in gypsum crusts from Antarctica, *Sphingomonas* sp. strain eh2 (GenBank accession no. AF548567, 92% sequence similarity).

3.3. Fluorescent In-Situ Hybridization (FISH) analysis

The community structure and seasonal shifts of the planktonic bacteria and archaea were further confirmed by performing FISH analysis. The bacterial-group-specific and archaeal-domain-specific oligonucleotide probes which were applied are summarized in Table 1. These probes were chosen based on the sequence information of the dominant bacterioplankton present in the Lake Jöri XIII. Water samples taken from different depths and seasons were used for the direct abundance analysis, without the need to either cultivate the cells or extracting their DNA.

The total cell densities determined by DAPI staining were lowest at the end of the ice cover period, i.e. 3.97 x 10^5 cells ml^{-1} in the upper water layer, 4.46 x 10^5 cells ml^{-1} in the middle of the water column and 4.73 x 10^5 cells ml^{-1} in the bottom of the lake, respectively. The bacterioplankton densities increased and reached their maximal abundances in summer with densities ranging from 1.68 x 10^7 to 1.91 x 10^7 cells ml^{-1}, whereas the densities in autumn varied between 1.67 x 10^7 and 1.75 x 10^7 cells ml^{-1}. Filamentous and large rod shaped cells (which have rarely been detected at the end of the ice cover period) bloomed during these periods and they can be visualized only by DAPI staining and the EUBI-III probe. They showed high abundances in the samples taken from all depths; however the lower water layers showed the highest cell numbers. In winter, the microbial cell densities decreased, with abundances ranging from 8.55 x 10^5 to 9.11 x 10^5 cells ml^{-1}. DAPI-stained filamentous cells still appeared frequently, especially in the samples from lower water layers.

The FISH analysis depicted in Fig. 5 summarizes the spatial and seasonal changes of the microbial community composition present in Lake Jöri XIII. The percentages of DAPI-stained cells that can be visualized with EUB I-III and ARCH915 probes were [62.0%; 1.4%] [percentage of eubacterial cells;

percentage of archaeal cells] at the end of the ice cover period in water just beneath the ice cover. The percentages of both, eubacterial and archaeal cells, increased in the lower part of the water column. In the middle of the water column the corresponding numbers were [70.2%; 3.1%], and in the bottom layer were [70.1%; 8.5%], respectively. During the summer when the water masses were mixing frequently, we detected [55.2%; 2.7%] in the surface water layer, [56.1%; 2.8%] in the middle and [62.6%; 5.9%] at the lake bottom. In autumn the corresponding numbers ranged from [52.7%; 3.2%] to [62.3%; 2.6%]. In winter, when the proportion of *Gram positive, High G+C* bacteria increases at all depths the following distribution was found: [60.9%; 1.0%] in the surface water layer, [63.4%; 3.1%] in the middle of the water column and [67.2%; 2.9%] at the bottom of the lake.

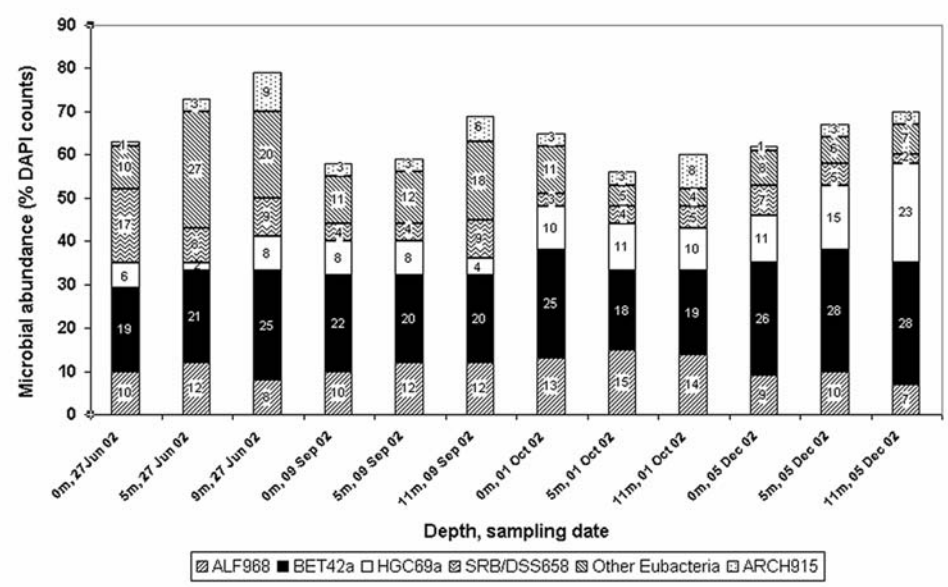

Figure 5. Enumeration of the microbial community composition in the water column of Lake Jöri XIII by FISH analysis*).

*) Percentage of hybridized cells in relation to total detected DAPI counts. Autofluorescent cells and NON EUB338 cells were subtracted from the total number of fluorescent bacterial cells (excluding Archaea). 'Other bacteria' refers to those bacterial cells (excluding Archaea) which hybridized by EUB338 but unaffiliated neither with ALF968, Bet42a, HGC69, nor SRB/DSS568.

The abundance and distribution of bacteria affiliated with the *beta*-subgroup of the *Proteobacteria* dominated the population in all samples. This finding reconfirms previous results obtained from comparison of clone sequences. Members of this subgroup were detected by the BET42a oligonucleotide probe, and showed abundances between 19% and 28% of total DAPI-stained cells (annual average 23%). At the end of the ice cover period, in summer and in autumn, the second most abundant group corresponded to the *alpha-Proteobacteria,* which were detected by the ALF968 probe. These dominances occurred at all depths, with values ranging from 8 to 15 % of the total DAPI-stained cells.

During the ice cover in early winter, the abundance of *Gram positive, High G+C* bacteria (*Actinobacteria*) showed higher values than the *alpha-Proteobacteria*. They reached 11% of the DAPI-stained cells (on the surface), 15% of the DAPI-stained cells (in the middle of the water column), and 20% of the DAPI-stained cells at the bottom of the lake. At the end of the ice cover period, during summer and autumn, the *Actinobacteria* were found at rather low abundances at all depths (2 to 11% of the DAPI-stained cells). The sulfate-reducing bacteria (detected by the SRB/DSS658 probe) were more abundant at the end of the ice cover period with an average of 11% (range, 8 to 17%) of the DAPI-stained cells. In summer, autumn and early winter the sulfate reducers made up only 5% of all DAPI-stained cells (range 2 to 9%).

Another remarkable result obtained with FISH was the high archaeal density in the lowest water layers of the lake. In samples collected on 27 June, 9 September, and 1 October, the densities of archaea reached their maxima of 9, 6, and 8%, respectively, of total DAPI-counts. Cells probed with the ARCH915 appeared as short rod and coccus morphotypes, whereas the filamentous-shaped archaeal cell could not be detected in these samples. Most of the cells were brightly fluorescent, a smaller portion showed dim fluorescence (Fig. 6).

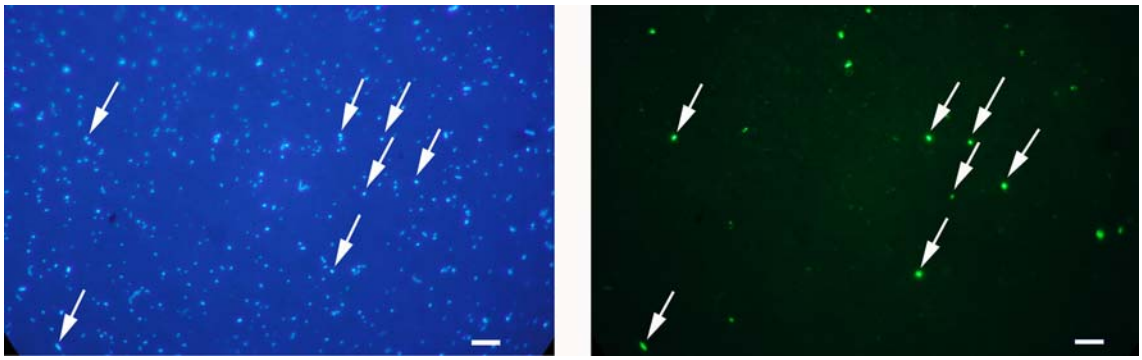

Figure 6. DAPI (left) and FISH (right) analysis of total cells and archaea present in samples collected towards the end of the ice covered period (May 31, 2003) Archaea were probed with the ARCH915 oligo-nucleotide probe.

12 different archaeal phylotypes resulted from a total of 104 clones selected by RFLP analysis (Table 5). The sequences revealed low levels of base identity compared to already existing sequences (similarity values ranged between 83 to 95%). The phylogenetic affiliation of the archaeal clone libraries to their closest relatives is depicted in Fig. 7. Over half of those were affiliated to methanogenic archaeal clones originating from habitats with various temperature conditions. Some sequences appeared to be clustered to the uncultured Euryarchaeota, but they had low nucleotide sequence similarity to sequences available in the Databank (less than 90% similarity) which generated deep branches in their phylogenetic affiliation. Some of them appear to present previously unrecognized archaeal taxa, which might have to be assembled in a new phylogenetic group.

Table 5. Phylogenetic grouping and identification of potentially novel archaeal clones from Lake Jöri XIII.

Clone designation, Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest species, accession no.	% Similarity	No of clones (percentage)
M01, AJ867612	788	Euryarchaeota; Methanocaldococcaceae	Clone ABS9, AJ227954	Anoxic sediment of rice field	91	*Methanocaldococcus indiensis strain SL43,* AF547621	86	11 (10.6%)
M02, AJ867613	850	Euryarchaeota; Thermococcaceae	Clone VAL31-1, AJ131276	Finnish forest Lake Valkea-Kotinen	94	*Palaeococcus helgesonii strain PI1,* AY134472	87	23 (22.1%)
M03, AJ867616; M10, AJ867614; M21, AJ867617; M36, AJ867618	794	Crenarchaeota	Clone SAGMA-D, AB050208	Waters from gold mines	99	*Cenarchaeum symbiosum strain B, AF083072*	88	48 (46.2%)
M06, AJ867615	788	Euryarchaeota	Clone pCIRA P, AB095126	Deep-sea hydrothermal vent	84	NONE	-	1 (0.9%)
M18, AJ867619	785	Euryarchaeota	Clone pCIRA P, AB095126	Deep-sea hydrothermal vent	84	NONE	-	2 (1.9%)
M19, AJ867620 M94, AJ867621	787	Euryarchaeota, Methanomicrobiaceae	Clone pMC2A35, AB019753	Deep-sea hydrothermal vent	83	*Methanoculleus thermophilus strain CR-1,* AB065297	83	2 (1.9%)
M22, AJ867622	805	Euryarchaeota	Clone 33-FL18A98, AF355814	Mid-ocean ridge sub sea floor habitat	87	*Thermococcus zilligii strain AN1,* U76534	85	1 (0.9%)
M24, AJ867623	790	Euryarchaeota; Methanospirillaceae	Clone VAL1, AJ131263	Finnish forest Lake Valkea-Kotinen	98	*Methanospirillum sp.,* L48407	95	5 (4.8%)
M33, AJ867624	790	Euryarchaeota	Clone pCIRA P, AB095126	Deep-sea hydrothermal vent	84	NONE	-	1 (0.9%)
M35, AJ867625 M52, AJ867626	814	Euryarchaeota; Methanothermaceae	Clone VAL31-1, AJ131276	Finnish forest Lake Valkea-Kotinen	88	*Methanothermus fervidus strain DSM2088,* M59145	86	6 (5.77%)
M98, AJ867627	788	Euryarchaeota, Methanospirillaceae	Clone 15ar18, AY251027	Freshwater sediment	98	*Methanospirillum sp.,* L48407	95	1 (0.9%)
M100, AJ867628	809	Euryarchaeota; Methanocaldococcaceae	DGGE band PSARC-2, AJ296597	Petit Saut Lake	79	*Methanococcus jannaschii strain DSM 2661,* M59126	89	3 (2.88%)

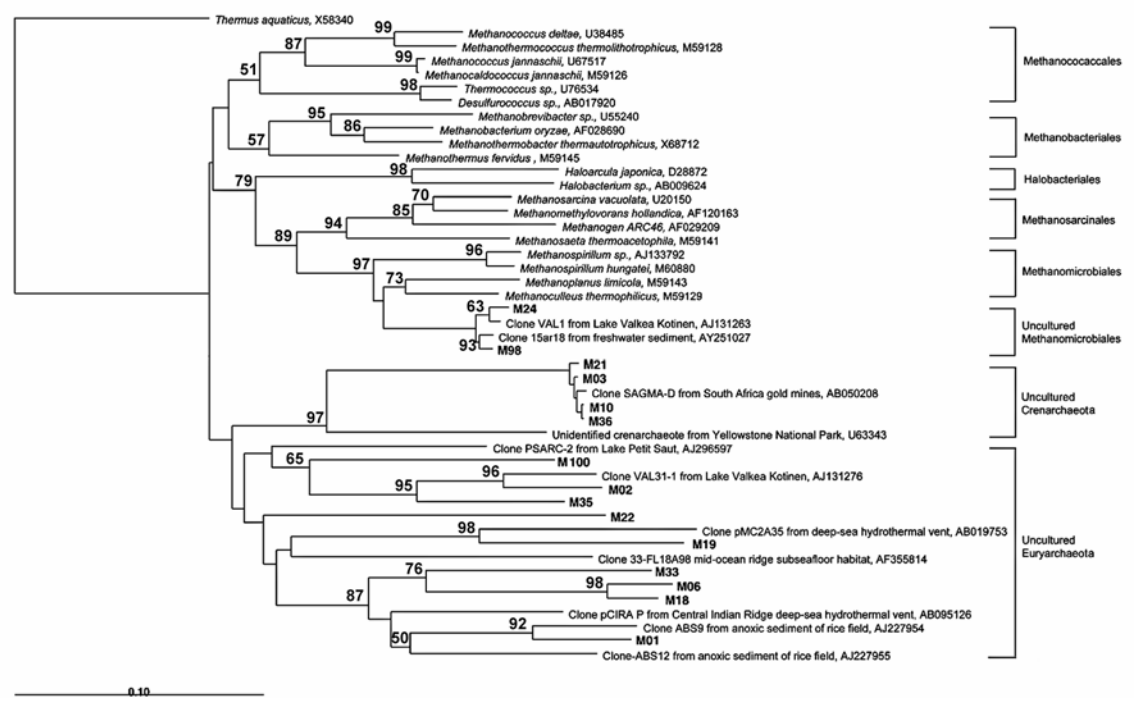

Figure 7. Phylogenetic affiliation of 16S rRNA gene sequences of the archaeal clones from Lake Jöri XIII and their most closely related neighbors. The 16S rRNA gene of the thermophilic bacterium *Thermus aquaticus*, X58340 of the bacterial class *Deinococci* is used as an outgroup organism. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar represents 10% sequence divergence.

The archaeal composition of the clone libraries from Lake Jöri XIII in relation to their closest known species is depicted in Fig. 8. All of the clones were very distantly related to any known species of the archaea. 48 clones (46.2 % of the total clone libraries) were affiliated to the clone SAGMA-D from South African gold mines with similarity value of 99%. Their known closest-species (88% sequence similarity) is a psychrophilic *Crenarchaeotum*, *Cenarchaeum symbiosum,* AF083072, an obligate symbiont of the marine sponge *Axinella mexicana* (Schleper et al. 1997). The rest of the clone libraries (still with low sequence similarities) are affiliated to euryarchaeota isolated from various thermophilic habitats (GenBank descriptions, unless otherwise noted). 22 clones (22.1% of the libraries) were 87% similar to *Palaeococcus helgesonii* strain PI1 (GenBank accession no. AY134472) a facultative anaerobic, hyperthermophilic archaeon from a geothermal spring on Vulcano Island, Italy. 11 clones (10.6%) were similar to *Methanocaldococcus indiensis* sp. strain SL43, a hyperthermophilic methanogen isolated from the Central Indian Ridge (with 86% sequence similarity). Two phylotypes represented by six clones (5.8%) were 95% similar to *Methanospirillum* sp., L48407, a methanogen isolated from blanket bog peat. Another six clones (5.8%) were 86% similar to an anaerobic, hyperthermophilic archaea *Methanothermus fervidus* strain DSM 2088 (GenBank accession no M59145) isolated from a hot solfataric spring in Iceland. Three clones (2.9%) were 89% similar to

Methanococcus jannaschii strain DSM 2661 (GenBank accession no M59126), a hyperthermophilic autotrophic archaea. *Methanococcus jannaschii* originated from sediments at the base of a "white smoker" chimney found at 2'600 m depth, at 21ºN on the east Pacific Rise. Two clones (1.9%) were similar to the *Methanoculleus thermophilus* (a synonym of *Methanogenium thermophilicum*) strain DSM 2373, AB065297, a thermophilic marine methanogenic bacterium. One clone (0.9%) was 85% similar to *Thermococcus zilligii* strain AN1, DSM 2770 (GenBank accession no. U76534), isolated from a terrestrial freshwater geothermal pool in New Zealand. This isolate differs from other *Thermococci* in its lower than usual optimal NaCl concentration (50mM or 0.29%) and generally lower optimal temperature for growth (75 to 80ºC) (Ronimus et al. 1997). Three phylotypes represented by 4 clones, which were 84 to 87% similar to the clone pCIRA-P from the Central Indian Ridge deep-see hydrothermal vent, were not affiliated to any known-species.

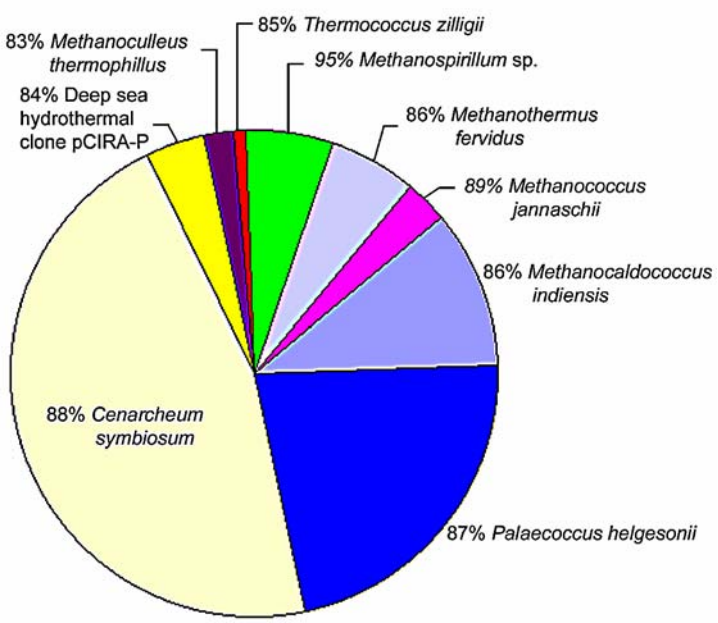

Figure 8. Archaeal composition of the clone libraries from Lake Jöri XIII and similarity values to their closest known-species.

4. Discussion

4.1. Environmental fluctuations in high mountain lake habitats

Cold extreme ecosystems are common on earth. They are dominant in the deep ocean, at high altitudes, i.e. high mountains and at high latitudes i.e. Arctic and Antarctic regions in snow, ice, water and permafrost soil. Ecosystems in these environments have normally little or no vegetation and consequently they are oligotrophic, and harbour a limited trophic complexity. High mountain lake ecosystems are habitats which are seasonally influenced by extreme environmental fluctuations. This is in contrast to Antarctic habitats with constantly low temperatures and restricted environmental variables (Ellis-Evans 1996). During the long ice-cover period, high mountain ecosystems are exposed to cold temperatures, nutrient deprivation, darkness below the thick ice cover, and anoxic condition caused by oxygen depletion at greater water depths. Towards the end of the ice cover period, oxygen concentrations are as low as 0.1 mg l^{-1} (2 to 4 µM, corresponding to less than 1% oxygen saturation at high altitudes) (Granados and Toro 2000). During the snow melt period cold, dense water enters the lake supplying oxygen to the deep water layers which can quickly change oxygen concentrations to approximately 10 mg l^{-1} in the entire water column (Granados and Toro 2000). During the short summer period, high mountain habitats are exposed to high levels of solar UV radiation, high diurnal and sudden temperature changes, deep light penetration, and increasing nutrient concentrations. The oxygen concentration during this time varies between 7.6 mg l^{-1} and 11.1 mg l^{-1}) (Wille et al. 1999).

Ecosystems exposed to strong environmental fluctuations provide heterogeneous ecological niches. Our study is based on the hypothesis that the microbial community adapts itself by continuously selecting for those members which are best adapted to the predominant conditions in the ecosystem. This selective adaptation model is derived from an idea proposed by Elena and Sanjuán (2003). Different community ecotypes which are either niche specialists or niche generalists respond to environmental fluctuations by increasing their rate of propagation. The observed changes in community composition are thus a consequence of strongly fluctuating population sizes. Most microorganisms present in habitats regulated by strong and frequently changing dominants are *r*-strategists, i.e. organisms which respond to environmental changes by increasing or decreasing their growth rates. The diversity of different ecotypes increases adaptation fitness which guarantees stability and drives the evolution of an ecosystem under extreme and extremely variable conditions. A small number of organisms present in the Jöri Lake XIII also adopt a K-strategy. A high rate of propagation is less advantageous for these organisms than the ability to adapt their life styles to a number of different conditions. They become the generalists which appear under more than one set of ecological determinants. K-strategic niche specialists are less suited to guarantee proper community responses in high altitude habitats with frequently changing conditions since their ability to adapt to niches with different ecological conditions is regarded as small (Buckling et al. 2003).

4.2. Spatial and seasonal community composition changes as revealed by TTGE analysis

The seasonal variations in the microbial compositions are linked to the changes in the physicochemical determinants (Fig. 1, Table 3) and seasonal nutrient fluctuations (Fig. 2). Temperature appears to be the major determinant. The instability of the water masses in Lake Jöri XIII is due to temperature fluctuations which influence the vertical distribution of the bacterioplankton. During the thermally stable stagnation period, TTGE patterns showed distinct profiles for different depths (Fig. 3).

Similar community changes between upper and bottom layers of the water columns were also found in meromictic lakes, for example, Lake Vilar in north-eastern Spain (Casamayor et al. 2002) and in the alkaline Mono Lake (Hollibaugh et al. 2001). Although the conditions in the upper mixolimnion, in deeper layers of the chemocline and in the monimolimnion are very different in these lakes, the community structures in the more stable water layers, specifically in the anoxic monimolimnion, were very similar for samples collected over the year. This was not the case for the oxic zone of the mixolimnion (Hollibaugh et al. 2001), in which the community structures changed dynamically over the annual sampling period.

Under homothermic conditions in summer and autumn, when the water masses were displaced by water turbulences, the TTGE patterns were very similar at all depths (Fig. 3). A different case has been previously reported in the alpine meromictic lake Cadagno (Egli et al. 2004). This lake shows a permanent stratification, chemically as well as physically, as represented by an oxic mixolimnion, a chemocline water column and an anoxic, high salinity monimolimnion. There, the spatial distribution of phototrophic bacteria present in a dense layer changed diurnally by active vertical movement in response to the light conditions and the chemical gradient (Egli et al. 2004).

The combination of molecular approaches i.e. TTGE, PCR-assisted cloning and sequencing allowed us to describe and identify the diversity and community succession in this habitat. The sequence identification of the most abundant bacterioplankton in Lake Jöri XIII revealed that 32 out of 45 sequences (84% of all the sequences analysed) belong to bacteria with an r-strategy life style. 13 other bands (representing 6 different sequences) which were present during several seasons would count as K-strategic generalists. Examples for K-strategists are the two clones representing organisms closely related to *Gemmatimonas aurantiaca* strain T-27T, i.e. band A1 (shared with band B1, 97% similarity to *G. aurantiaca*) and band C1 shared with band D1 (90% similarity) which appeared at all seasons. The communities consist of more niche specialists than generalists, which support the hypothesis that microbial populations in this ecosystem have a high ecophysiological flexibility. In addition, the large number of unique TTGE bands indicates that microbial populations in Lake Jöri XIII are phylogenetically diverse which gives the community an enormous potential to dynamically respond to fluctuating environment conditions.

The most abundant bacteria in each particular season belong to diverse taxonomic groups (Table 4, Fig. 4a and 4b). This indicates that different microbial communities can play various ecological roles under particular conditions. Although it is difficult to infer physiological function from diversity alone, in some cases, the likely ecophysiological properties can be inferred by considering the physiology abilities of the closest known-relatives. We identified the presence of a 16S ribosomal RNA gene for the chloroplast of the green algae *Chlorella saccharophila* strain 3.80 (GenBank accession no. D11348). It is not surprising that the presence of this phototrophic organism (bands B7 and C9) was found during summer and autumn mixing events, but that was absent during the ice cover period. Some of the predominant bacterioplankton organisms for which only distantly related to known-species (less than 90% sequence similarities) make the prediction of their ecological functions more difficult.

Some of the bacteria in the lake play important ecological role in iron cycling. These bacteria are represented by the bands A5, C6, D6 and D4. The first 3 bands have different sequences, but all are 97 to 98% closely related to *Rhodoferax ferrireducens* (GenBank accession no. AF435948). This bacterium is a psychrotolerant, facultative anaerobic bacterium with the ability to oxidize acetate and reduce Fe (III). It was first isolated from anoxic sediments (Chaudhuri and Lovley 2003). In a recent study Chaudhuri and Lovley (2003) reported that this novel bacterium possesses the ability to oxidize glucose to CO_2 linked to Fe (III) reduction. The next relative to the clone represented by band D4 was *Leptothrix cholodnii* which was found to be dominant at the beginning of the ice cover period. These bacteria are probably also involved in anaerobic Fe (III) respiration and aerobic Fe (II) oxidation, respectively.

In an *in vitro* experiment, Rainey and Travisano (1998) showed that the phenotype diversity increased more rapidly in cultures kept in a heterogeneous environment than those incubated under homogeneous conditions. This corroborates our observation about the dominance of niche specialists for particular conditions while some niche generalists, for example, the ones represented by A1, A10, A11, B7, B9, and C1 had evolved to competition fitness for fluctuating environments. Niche specialists have lost their ability to quickly adapt to alternative niches with heterogeneous ecological conditions (Buckling et al. 2003, Elena and Sanjuán 2003).

The sequences of the predominant bacterial clones depicted in the phylogenetic relations (Fig. 4a and 4b) demonstrate that many of them are closely related to bacterial clones originating from geographically widely distributed cold ecosystems, e.g. from Austrian Alpine Lake Gossenkölle, from southern hemisphere glaciers, from Antarctic cryoconite holes, Antarctic microbial mats, Arctic Sea ice, and Arctic Lake Toolik. In addition, a large number of the sequences were also affiliated to the clones isolated from various other aquatic habitats, e.g. Lake Fuchskuhle, Lake Loosdrecht, Weser estuary, Elbe river, Wulfbach river, ground water, waste water treatment, and crater lake, whereas only a limited number of the closest neighbours originated from terrestrial habitats. These results are consistent with previous studies (Glöckner et al. 1999; Glöckner et al. 2000).

In our study we did not find *gamma-Proteobacteria*. This result differs from our experience in isolating heterotrophic bacteria from this habitat (Chapter 2) in which the *gamma-Proteobacteria* appeared in enrichments on low-nutrient agar medium. The culture-independent result is consistent, however, with previous studies which reported low abundances of *gamma-Proteobacteria* in high mountain lake habitats. The abundance of the *gamma-Proteobacteria* detected by FISH was only about 1 to 2 % in slush, snow, and lake water (Alfreider et al. 1996; Pernthaler et al. 1998) and less than 7% (Glöckner et al. 1999) of the total density. Cultivation techniques, in which this subgroup appears prominently (Glöckner et al. 1996), might significantly shift the microbial community structure through enrichment selection.

4.3. Community composition dynamics confirmed by FISH

FISH was used to determine the spatial and seasonal abundance in community structure, because PCR-based methods alone might produce bias (Suzuki and Giovannoni 1996). FISH is a powerful tool which enables one to visualize and quantify specific phylogenetic groups by using appropriate rRNA-targeted oligonucleotide probes for studying changes in community composition (Amann et al. 1995). This method can include bacteria which may resist the DNA extraction step in PCR-based methods and it provides the possibility to monitor the dynamic changes of a unique or specific endogenous population by using a single 16S rDNA sequence retrieved from the site (Pernthaler et al. 1998).

In our analysis, FISH confirmed the results from sequencing of the 16S rDNA of the clones representing the predominant TTGE bands. The spatial and seasonal dominance of the *beta-Proteobacteria* could be demonstrated in all samples analysed (Fig. 5). This finding is consistent with previous studies reported for other high mountain lake habitats and its snow cover (Alfreider et al. 1996; Pernthaler et al. 1998) and other freshwater ecosystems (Glöckner et al. 1999). The differences which we detected between the cloning and the FISH approach refer to the sulfate reducing bacteria which were detected by using FISH, but which did not appear in our clone libraries.

Microbial shifts from the end of the ice cover period in June to the late summer season in September were characterized by a decreasing number of sulfate reducing bacteria and an increase in the population of *Gram positive, High G+C* bacterial populations. Distinct successions in community composition were demonstrated again between autumn and early winter, when the *beta-Proteobacteria* and *Gram positive, High G+C bacteria* became the two most dominant bacteria in the community, whereas the abundance of the *alpha-Proteobacteria* populations declined.

4.4. Archaeal diversity in Lake Jöri XIII

A remarkable FISH result was the high abundances of Archaea detected in the lowest water layers, especially at the end of the ice cover period (Fig. 5 and Fig. 6). Previous studies reported the presence

of archaeal clones in cold habitats (Fuhrman et al. 1992; Hershberger et al. 1996), but little is known about archaeal diversity from cold high mountain lakes. In an effort to more precisely identify the archaea from this environment, we did cloning of the 16S rDNA from the total community and constructed archaeal clones libraries.

Approximately 52% of our archaeal clones were closely related (98 to 99% sequence similarity) to known sequences from clones observed in temperate habitats. They fell into 3 lineages, i.e. clones represented by M03, M24 and M98 (Fig. 7, Table 5). The remaining clones were only distantly related to any known sequences, and they formed deeply branching affiliations, with levels of identity between 79 and 94%. This finding may indicated that the novel archaeal sequences belong to unknown groups or represent currently unrecognized phylogenetic groups. It was surprising that these novel high mountain archaea were closely related to the clones originating from geographically distant, greatly different altitudes or to those characterized as high temperature phylotypes originating from deep sea hydrothermal vents, sub-sea floor habitats, and the hot springs of Yellowstone National Park (Fig. 7 and Fig. 8). These results differed from what was found among the predominant bacterioplankton, in which many organisms were affiliated to clones from diverse cold ecosystems. To our knowledge there was no previous report which reported similar observations in high mountain lakes. Therefore, we would like to propose a new candidate archaeal phylogenetic group.

Many of the Lake Jöri XIII archaeal clones were phylogenetically affiliated with other archaea originating from methane-rich habitats. Efforts to isolate the previously-uncultivated archaea from this habitat and to study their physiological properties, as well as to characterize their environmental requirements would be a challenging subject for future studies.

Despite of commonly low temperature condition in the habitat from which we obtained our archaeal clones, presently their closest relatives correspond to methanogenic archaeal clones retrieved from high temperature habitats. This is not all that uncommon; the presence of low temperature-adapted methanogenic archaea was also previously recovered from Ace Lake in Antarctica (DeLong et al. 1998). The new psychrotolerant or psychrophilic group of archaea might belong to those that contain the novel membrane lipid "crenarchaeol" (Schouten et al. 2000). This unusual lipid is a glycerol dibiphytanyl glycerol tetraether (GDGT) which contains one cyclohexane and four cyclopentane rings formed by internal cyclisation of the biphytanyl chains. This might confer the ability to live at low temperatures (Sinninghe Damsté et al. 2002).

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High mountain snow communities: habitats at lower altitudes reveal a higher diversity than those at higher altitudes

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Abstract:

The microbial diversity of the snow communities from two different high altitude habitats above the tree line of the Swiss Alps, the Jöri lakes catchment (at altitudes between 2'489 m a.s.l. and 3'060 m a.s.l.) and Jungfraujoch (3'500 m a.s.l.) were investigated employing PCR-based techniques.

The diversity of the snow communities from the two altitudes was quite different. The most diverse snow communities were present in the colored snow from sites at lower altitude (Jöri lakes catchment), which contain *Chlamydomonas* blooms. The blooming was induced during snowmelt in June by the high exposure to solar radiation and the availability of liquid water. The Shannon–Weiner (H') indices derived from universal clone libraries of red, white and brown snowfields from the Jöri lakes catchment were 2.47; 2.02, and 1.58, respectively. The presence of primary producers in snow habitats enriched the community of prokaryotes. Higher altitude snow habitats which are permanently frozen (Jungfraujoch, JFJ) do not allow for algal blooms to develop; they revealed lower H' indices of 0.51. H' values from JFJ ice samples, however, showed H' of 2.02 and 1.18 from universal and bacterial libraries, respectively. It is interesting, but not surprising, that some of the sequence from JFJ snow habitat, which is easily accessible by tourists, corresponded to bacteria of human-origin.

Snow cover communities in high mountain habitats harbor prokaryotic and eukaryotic phyla. The majority of clones from Jöri snow belong to the bacterial domain, mostly to the *beta-Proteobacteria* subgroup. In JFJ snow habitats, *Actinobacteria* were dominant in air isolates and in the entire snow community, but we also obtained clones corresponding to Crenarchaeota that were not present in snow from the lower altitude snow habitats. In JFJ ice habitats, the *beta-Proteobacteria* subgroup was the most abundant. Our results suggested that microorganisms transferred via the atmosphere from distant locations might influence the diversity of remote snow habitats at high altitudes.

1. Introduction

High altitude areas in the European Alps are periodically influenced by Saharan dust from episodic long-range-storms (De Angelis and Gaudichet 1991; Psenner 1999). Air borne Saharan dust is thus an important component of the snow, which is deposited at high altitudes. Annual precipitation in these areas can lead to snow covers as thick as 1 to 4 m (Psenner and Sattler 1998). Airborne dust particles are a constant source of microbial cells, pollen, plant debris, insects, and other organisms of terrestrial-origin. Dust depositions have also been reported as essential sources of ions and nutrients for alpine lakes (Psenner 1999). Dust events play essential roles in providing allochthonous nutrients for organisms present in these cryo-habitats. Under certain conditions, they can develop a rich diversity of prokaryotic and eukaryotic microorganisms (Felip et al. 1995).

Based on its physical characteristics, the snow cover at high altitudes is an extreme habitat. Nutrients and liquid water are commonly limiting, there is a strong exposure to UV radiation, the temperatures in winter are constantly below freezing and there are frequent harsh weather fluctuations. The extreme conditions provide a natural selection system for well-adapted organisms. Airborne communities are quickly fragmented into organisms which can survive and sometimes propagate, those which can convert into long-lasting resting stages, and those which will not survive at all.

Despite of the harsh conditions, recent studies imply that the slush layers i.e. the melting snow cover, and glacier ice can harbor high diversity and activity of microbes thriving at freezing temperatures (Felip et al. 1995; Alfreider et al. 1996; Psenner and Sattler 1998; Skidmore et al. 2000). The heterotrophic microbial communities found in snow cover differed from those found in lakes of the catchment (Felip et al. 1995). These results indicate that snow and ice habitats are easily colonized by allochthonous microorganisms originating from far away, whereas high mountain lakes harbor established autochthonous communities.

Snow communities contain prokaryotic and eukaryotic organisms. Although fresh snow covers are white, during the melting period, their color can change into shades of red, orange, brown, green, or even bluish. Mass developments (blooming) of pigmented eukaryotic algal cells and cyanobacteria are responsible for these natural phenomena, which have been known for a long time (Madigan et al. 2003). Microscopic analysis has revealed an abundance of microbial cells, among them ciliates, fungi, autotrophic and heterotrophic flagellates, etc. all inhabiting snow cover habitats.

The water volume contribution of the annually melting snow cover for shallow mountain lakes can be considerable. Hence, the composition and processes occurring in the snow cover can significantly influence the nutrient budget and population composition in these ecosystems (Felip et al. 1995). In late spring, melt water feeds the lake with the weathering materials from rocks and soil, with air borne dust particles, debris and living organisms from the snow cover. All of these materials affect the solute content in the lake water and increase the nutrient contents, which show bursts during the melting season (Iqbal- Nava 2003).

The microbial snow communities appear to contain a diversity of unknown microorganisms. Recent studies provided information about the abundance, activity, and the community composition (Felip et al. 1995, Alfreider et al. 1996, Pernthaler et al. 1998, and Glöckner et al. 1999). However, snow communities from high mountain snow habitats were little studied so far, with regard to their rDNA sequence affiliation and phylogeny. Here we report on comparative studies on the diversity of microorganisms in snow and ice habitats from different altitudes as revealed by 16S-rDNA and 18S-rDNA analysis. PCR-assisted cloning, Restriction Fragment Length Polymorphisms (RFLP), and sequencing techniques have been used to assess the microbial community composition. With these approaches, a large number of not yet cultured microorganisms from diverse origins were discovered in snow and ice habitats.

Organisms that actively grow at near freezing temperatures and low nutrient concentrations normally face a number of growth limiting constraints, such as decreased enzyme reaction rates, lowered uptake and transport processes due to less permeable membranes (Feller et al. 1996; Graumann and Marahiel 1996). Studying microorganisms present in cold ecosystems will not only uncover their phylogenetic relatedness and their ecological role in the environment, they may also lead us to understand living strategies under a variety of harsh conditions.

2. Materials and Methods

2.1. Sampling sites and sample collection

Fresh snow samples were taken from 2 different altitudes of the Swiss Alps. Snow samples from the lower altitude habitat were represented by snow of the Lake Jöri XIII and nearby areas in the Jöri catchment. This catchment is located in the south eastern Swiss Alps (46º46'N/ 9º58'E), at altitudes between 2'489 m and 3'060 m a.s.l. Lake Jöri XIII is a young deglaciated lake, one of 22 small and middle-size lakes; it is situated at 2'640 m a.s.l. The Jöri catchment is snow-covered between November/December and July, but the area is completely snow-free every year for a few months, normally by the beginning of August.

Samples from higher altitude sites were represented by snow and air samples collected from the Mönchsjoch snowfield near the Jungfraujoch (JFJ) research station (Swiss Alps, 46°32' N/ 7°59' E, approximately of 3'500 m a.s.l.). The study site has an average air pressure of approximately of 653 mbar and an average air temperature of minus 8°C (data of High Altitude Research Station at JFJ). This guarantees a permanent snow cover at all seasons. The air temperature at the sampling date was minus 0.4°C and the air pressure was 674.4 mbar. Samples were collected from the snow pack as well as from a 1 m ice core below. Neither of the sites contains an appreciable vegetation cover. The JFJ site lies in permanent ice, whereas the Jöri site lays about 1'000 m above the upper tree line and is surrounded by barren rock with very little soil cover.

Jöri samples, which consisted of brown, red, and white snow, were collected in May 2003, whereas the white snow at JFJ and air samples were collected in November 2003. Brown snow sample was collected from surface snow along the shore of Lake Jöri XIII when it was still fully ice-covered. The red and white snow samples were collected from snowfields in the lake catchment. The red snow sample was taken as a 5-mm thick snow surface layer, whereas the white snow sample was taken from the surface of a not obviously coloured area. All snow samples were collected aseptically into sterile bottles. Samples were kept at low temperature in a cooling box during transport to the laboratory.

Samples from the JFJ snow pack were collected in sterile plastic tubes (63 mm inner diameter, 60 cm long), capped and allowed to thaw over night in the 4°C room. The ice core (90 mm in diameter, 1

m long) was subsampled and samples were stored in sterile plastic bags. Air isolates from JFJ were collected aseptically by using an air germ collector type MAS100-Eco (Microbiology and Bioanalytic, MBV AG, Zürich). Air was blown directly onto plates containing agar medium (pH 7.2) with the following ingredients: bacto tryptone 5 g l^{-1}, yeast extract 10 g l^{-1}, NaCl 5 g l^{-1}, bacteriological agar 15 g l^{-1} and cyclohexymide (50 μg/ml) to prevent the growth of eukaryotic organisms. About 140 liters of air were allowed to impact a plate (mass normalized to sea level pressure of 1'000 mbar). Plates were incubated at room temperature (23ºC±1) for 2 days. Colonies were subcultured onto new medium. We randomly selected 46 air isolates for DNA extraction.

2.2. Microscopic analysis of microbial communities

Bright field and epifluorescence micrographs of the snow communities were taken with a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen Germany) connected to an Optronic video camera. Chlorophyll autofluorescence of the snow algae was observed with an excitation wavelength of 450-490 nm and a cut off filter of 510 nm. For the observation of the smaller microbial cells, a DAPI staining was performed. The snow samples were filtered on GTTP Millipore filters, and the DAPI-stained cells were observed by using the UV filter block (excitation wavelength 365-395 nm, cut off filter 395 nm). Electron micrographs were made with the Scanning Electron Microscope Model S-4100 (Hitachi Tokyo Japan) after the cells were fixed with 2% glutaraldehyde at 4ºC overnight.

2.3. Nucleic acid extraction

Different volumes were used for the extraction of total genomic DNA (Table 1). 150 ml water from melted white snow and ice samples were used. For the brown and red snow samples, which contained more cells and particles, 50 ml water from melted snow were filtered aseptically (filter type GVWP, 0.22 μm pore-size, Millipore). The filters were placed in sterile 1.5 ml tubes; the cells on the filters were washed with lysis buffer and further processed according to the methods recommended by the manufacturer (Qiagen Corp., Stanford, USA). 3.0 ml culture grown overnight in liquid LB medium was used to extract genomic DNA from the pure isolates and treated by the same methods as for the snow samples.

Samples were treated with the protocol for Gram positive bacteria, which also allows extracting DNA from Gram negative bacteria. An additional cell lysis step was applied after lysozyme treatment. We used 100 mg glass beads (diameter 0.25 mm) for disrupting cells by bead beating. The tubes were shaked at 80% maximum speed in a model MM2000 bead-beater (Retsch, Haan, Germany) for 1 min. After bead-beating, samples were further processed according to the protocol recommended by the manufacturer (Qiagen Corp., Stanford, USA). DNA extracts were checked by electrophoresis using a

1% agarose gel and stained in a 1 μg ml^{-1} ethidium bromide solution. The tubes containing extracted genomic DNA were stored at -20°C until further processing.

Table 1. Total community genomic DNA sources used in this study, primers used for cloning and overview of the clone analysis.

Origin	Sample source for environmental genomic DNA extraction	Primer used		No. clones obtained	No. clones analysed	No. Unique RFLP
		Forward	Reverse			
Red snow from nearby area of Lake Jöri XIII	50 ml of melted-snow	S-D-Bact-0008-b-S-20	S-D-Bact-1524-a-A-18	74	74	19
		S-D-Euk-0008-b-S-16	S-D-Euk-1512-a-A-22	8*	8	1
		S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	81	64**	21
Brown snow cover from Lake Jöri XIII	50 ml of melted snow	S-D-Euk-0008-b-S-16	S-D-Euk-1512-a-A-22	6*	6	5
		S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	126	64**	15
White snow from nearby area of Lake Jöri XIII	150 ml of melted snow	S-D-Euk-0008-b-S-16	S-D-Euk-1512-a-A-22	5*	5	3
		S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	58	24**	11
White snow from Jungfraujoch	150 ml of melted-snow	S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	72	72	9
		Arch-0089-F	Arch-0915-R	31	31	6
Ice core from Jungfraujoch	150 ml of melted-ice	S-D-Bact-0008-b-S-20	S-D-Bact-1524-a-A-18	66	66	8
		S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	38	38	10
Jungfraujoch isolates	46 isolates (pure cultures)	S-*-Univ-0519-a-S-18	S-*-Univ-1392-a-A-15	-	-	6

*More colonies did not have inserts; they appeared as blue colonies on the LB selection medium containing X-gal+Ampicillin (50 μg l^{-1}) + Kanamycin (50 μg l^{-1}).

**Randomly selected clones.

2.4. Polymerase Chain Reaction (PCR) and cloning

Different primer pairs were used for total community rDNA analysis (Table 2). Primer pairs targeting the small sub unit (ssu) rDNAs of the domains of *Bacteria*, *Archaea*, *Eukarya* and eukaryotic chloroplasts and mitochondria were used. The general bacterial primers S-D-Bact-0008-b-S-20 and S-D-Bact-1524-a-A-18 were applied to amplify nearly the full-length of the 16S rDNA of the total bacterial community from snow isolates (Table 2).

PCR reactions were carried out in a total volume of 25 μl, containing a mixture of (final concentrations) *Taq* buffer (1x) (Invitrogen), 2.0 mM $MgCl_2$ (Invitrogen), 0.1 mg ml^{-1} Bovine Serum Albumin DNase-free (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTPs, of 200 nM each primer, 40 U ml^{-1} *Taq* Polymerase (Invitrogen), dH_2O, and approximately 50-100 ng template DNA. PCR was performed with a Techne Thermocycler (Techne LTD, Duxford Cambridge, UK).

The following PCR program was used for eukaryotic ssu rDNA amplification: initial denaturation at 94ºC for 90 sec, 20 cycles of 94ºC for 20 sec, 52ºC for 30 sec and lowering the temperature by

0.2ºC in every cycle, elongation temperature at 72ºC for 80 sec. These steps were followed by another 15 cycles of 94ºC for 20 sec, 49ºC for 30 sec, 72ºC for 80 sec, increasing the period by 1 sec every cycle and a final extension step at 72ºC for 10 min.

Table 2. List of oligonucleotide primers used in this study.

No.	Oligonucleotide	Usage	Sequence (5'- 3')	*E. coli* position	Target	Reference (s)
1.	S-D-Bact-0008-b-S-20	PCR Primer, cloning, sequencing	AGA GTT TGA TCM TGG CTC AG	16S (8-27)	Bacterial domain	Lane, 1991; Hicks et al, 1992
2.	S-D-Bact-1524-a-A-18	PCR Primer, cloning, sequencing	AAG GAG GTG ATC CAR CCG	16S (1524-1541)	Bacterial domain	Lane, 1991
3.	S-*-Univ-0519-a-S-18	PCR Primer, cloning, sequencing	CAG CMG CCG CGG TAA TWC	16S (519-536)	Universal	Lane, 1991
4.	S-*-Univ-1392-a-A-15	PCR Primer, cloning, sequencing	ACG GGC GGT GTG TRC	16S (1392-1406)	Universal	Lane, 1991
5.	S-D-Euk-0008-b-S-16	PCR Primer, cloning, sequencing	TCY GGT TGA TCC TSC C	18S (8-23)	Eukaryotic	Horath, unpublished
6.	S-D-Euk-1512-a-A-21	PCR Primer, cloning, sequencing	AC GGH TAC CTT GTT ACG ACT T	18S (1512-1532)	Eukaryotic	Horath, unpublished
7.	Arch-89F	PCR primer, cloning, sequencing	ACG GCT CAG TAA CRC	16S (89 –103)	Archaeal domain	Hershberger et al, 1996
8.	Arch-915R	PCR primer, cloning, sequencing	GTG CTC CCC CGC CAA TTC CT	16S (915 –934)	Archaeal domain	Stahl and Amann, 1991
9.	S-*-Univ-0519-a-A-18	Intermediate primer for sequencing	GWA TTA CCG CGG CKG CTG	16S (519-536)	Universal	Lane, 1991
10.	S-*-Univ-0519-a-S-18	Intermediate primer for sequencing	CAG CMG CCG CGG TAA TWC	16S (519-536)	Universal	Lane, 1991
11.	S-D-Bact-1099-b-S-16	Intermediate primer for sequencing	GYA ACG AGC GCA ACC C	16S (1099-1114)	Prokaryotic	Lane, 1991
12.	S-D-Bact-1099-b-A-16	Intermediate primer for sequencing	GGG TTG CGC TCG TTR C	16S (1099-1114)	Prokaryotic	Lane, 1991
13.	M 13 F	PCR primer to retrieve the clone insert	GTA AAA CGA CGG CCA G	-	Vector plasmid pCR®2.1-TOPO	Invitrogen Corp., California, USA
14.	M 13 R	PCR primer to retrieve the clone insert	CAG GAA ACA GCT ATG AC	-	Vector plasmid pCR®2.1-TOPO	Invitrogen Corp., California, USA

Bacterial ssu rDNA and the universal ssu rDNA were amplified using the following PCR program: initial denaturation at 94ºC for 130 sec, followed by 10 cycles of 94ºC for 15 sec, 61ºC for 30 sec and lowering the temperature by 0.5ºC in every cycle, and elongation temperature at 72ºC for 80 sec. These steps were followed by another 20 cycles of 94ºC for 15 sec, 56ºC for 30 sec, 72ºC for 90 sec with increasing the period by 1 sec every cycle followed by a final extension step at 72ºC for 10 min. The archaeal ssu rDNA was amplified by using the following PCR program: initial denaturation at 94ºC for 90 sec, 30 cycles of 94ºC for 90 sec, 48ºC for 90 sec and 72ºC for 120 sec. The final step was an extension at 72ºC for 15 min. Positive and negative controls were performed with every PCR assay.

PCR product sizes were confirmed by 1% agarose gel electrophoresis in 0.5X TAE running buffer [45 mM Tris-acetate, (pH 8.3) and 4 mM EDTA]. The DNA in the gel was checked by staining with ethidium bromide (1 μg ml^{-1}) and photographing under UV. PCR products that appeared as a thick, single DNA band were directly cloned into vector plasmid pCR®2.1-TOPO (3.9 kb), using TOPO TA Cloning Kit (Invitrogen Corp., California, USA). The vector plasmid containing cloned DNA inserts were transformed into *Escherichia coli* cells TOP10, as recommended by the manufacturer.

2.5. Amplification of 16S rDNA and 18S rDNA inserts and RFLP (Restriction Fragment Length Polymorphisms) analysis

The 16S rDNA and 18S rDNA inserts from the positive clones were reamplified by PCR using M13F and M13R primers. The whole cell PCR was performed in a 25 μl total volume in a Techne Thermocycler (Techne LTD, Oxford Cambridge, UK). Each reaction mixture contained (final concentrations) *Taq* buffer (1x) (Invitrogen), 1.5-2.0 mM $MgCl_2$ (Invitrogen), 0.1 mg ml^{-1} Bovine Serum Albumin DNase-free (Amersham, Pharmacia Biotech Inc.), 0.2 mM dNTP's, 200 nM of each, forward and reverse primer, 40 U ml^{-1} of *Taq* Polymerase (Invitrogen), dH_2O and template DNA from the positive clones. The corresponding PCR programs described above were applied to amplify the inserts.

For RFLP analysis, 8 μl PCR products were double-digested with 1 U restriction enzyme *Hin*fI (5'-G/ANTC) and *Hae*III (5'-GG/CC) in a 10 μl total reaction volume. DNA fragments were separated using 6% polyacrylamide gels. 3 μl of samples from the 6 μl total volume digested-PCR products and 3 μl of standard loading buffer were loaded to the gels. The electrophoresis was carried out with a DCode Mutation Detection system (Bio-Rad Laboratories). The gels were run for 90 to 120 min at 120 volts (7.5 V/cm) with 1x Tris-Acetate-EDTA (TAE) buffer. After electrophoresis, the gels were stained in solution containing 1 μg ml^{-1} ethidium bromide for 2 min, destained for 10 min in dH_2O, and photographed under UV transillumination.

2.6. Sequencing of the 16S rDNAs from the clones.

PCR products of the clones representing unique RFLP banding patterns (amplified by M13F and M13R primer set) were purified through the microcon centrifugal filter devices (Microcon YM 100, Millipore, Bedford, Mass., USA). Almost full-lengths of bacterial 16S and 18S rDNAs, as well as partial lengths of eukaryotic 18S rDNAs and ssu rDNAs obtained with universal primers were bidirectionally sequenced using ABI Prism® Big Dye™ v2.0 (Applied Biosystems). Primers listed in Table 2 were used for the sequencing-PCR reactions for archaeal, bacterial and eukaryotic clone libraries.

A 10 µl-single sequencing PCR reaction contained 5 to 20 ng DNA template, 3 µl Big Dye (Applied Biosystems) and 3 µl of a 1.5 µM stock solution of each primer. The products were purified with Sephadex G-50 (Amersham, Pharmacia Biotech AB) and loaded onto the sequencer (Applied Biosystems, ABI Prism 3100 Genetic Analyzer). Sequencing results were analysed by the Sequencher program (Applied Biosystems, ABI Prism 3100 Genetic Analyzer). We used the CHECK-CHIMERA program of RDP (http://rrndb.cme.msu.edu) to identify possible chimeric sequences in the clone library

2.7. Phylogenetic analyses

The closest neighbours of the sequences were obtained by using the BLAST (Basic Local Alignment Search Tool) available from NCBI (http://www.ncbi.nlm.nih.gov/blast). The 16S rDNA sequences of representative clones possessing different closest neighbours and/or sequence similarity were used for phylogenetic analysis. These representative clone sequences and their closest neighbour sequences were imported into the ARB software environment (http://www.arb-home.de/). The new sequences were imported to the ARB database (release May 2002) and aligned automatically employing the Fast Aligner V1.03 of the ARB phylogeny software package (http://www.arb-home.de/). The alignment was subsequently corrected manually based on secondary structure information and the closest relatives as references. The aligned sequences were added to the consensus tree available in the ARB database and the distances were calculated by using the maximum-parsimony approach. The phylogenetic trees were constructed by the neighbour-joining method as provided in the ARB package. The resulting neighbor-joining trees were evaluated by bootstrap analyses based on 100 resamplings.

3. Results

3.1. Phenotype analysis of microbial snow communities

Bright field, fluorescence and scanning electron (SEM) micrographs of the snow samples showed a rich diversity of microbial cells (Fig. 1A to 1L). Brown and red snow samples revealed more diverse and more abundant eukaryotic cells than the white snow samples. In red snow, the eukaryotic cells were dominated by aplanospores of the snow algae *Chlamydomonas* sp. (Fig. 1B and Fig. 1C). These non-vegetative cells appeared as red and round cells, approximately 20 to 50 micrometers in diameter (Fig. 1B). Observation by fluorescence microscopy showed that the aplanospores posses a bright red autofluorescence when excited by 450-490 nm light and a weaker red autofluoresence when excited by 365-395 nm light. The spores tend to stick to each other as well as to glass slides. Single spores were

surrounded by an unidentified gelatinous-sticky coat material. Squeezing the aplanospores produced an orange liquid.

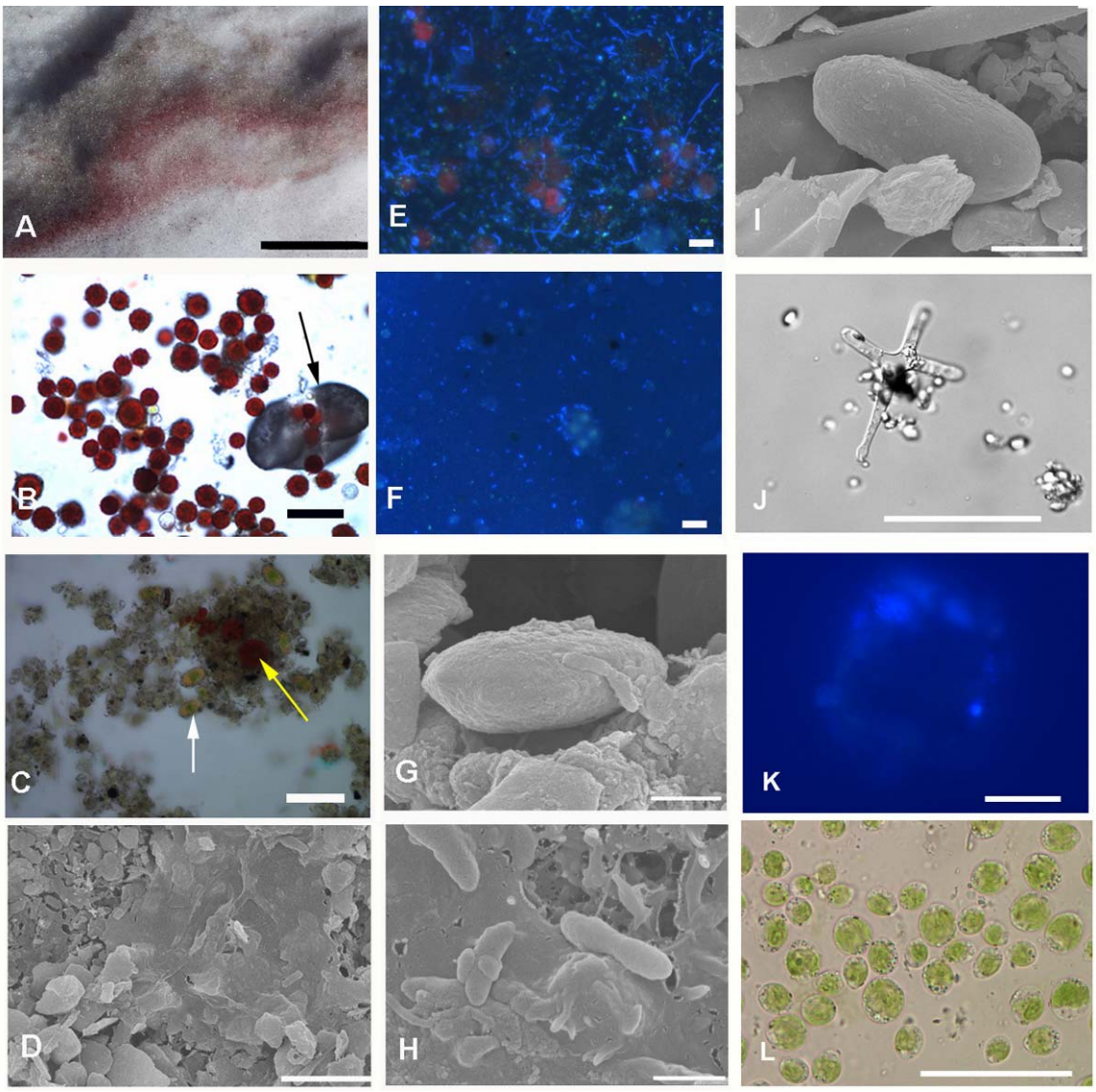

Figure 1. Red and brown snow microbial communities found in habitats nearby Lake Jöri XIII.

A. Red and brown snow cover found in Lake Jöri XIII habitat (bar = 15 cm). B. Bright field micrograph of red snow sample showing aplanospores of *Chlamydomonas* sp. and a pollen grain (black arrow) (bar = 50 µm). C. Bright field micrograph of brown snow sample showing *Chloromonas cryophila* cells (white arrow) and aplanospores of *Chlamydomonas* sp. (yellow arrow) (bar = 50 µm). D. Film-like structures and high abundance of mineral particles present in snow samples (bar = 10 µm). E and F. DAPI-stained bacterial cells of red snow (E) and brown snow communities (F), respectively (bar = 10 µm). G, H, and I. Scanning electron micrographs showing the small and large cells of snow microbial communities. Dividing cells (G, H) on the surface of film-like structures; all samples from snow habitat nearby Lake Jöri XIII (bar = 2 µm in G and H, 5 µm in I). J. Bright field micrograph showing a branching fungal mycelium (bar = 50 µm). K. A *Chlamydomonas* sp. aplanospore sorrounded by DAPI stained bacterial cells (bar = 10 µm). L. Bright field micrograph showing *Chloromonas nivalis* cells. *Chloromonas nivalis* is a unicellular green alga, motile, biflagellate, with an oval to round cell shape (bar = 50 µm).

A high abundance of mineral particles and film-like structures (Fig. 1D) were detected especially with the coloured snow samples. We also observed a high diversity of DAPI-stained much smaller cells that appeared as single cocci, rods or filamentous cells in the fluorescence microscope (Fig. 1E and Fig. 1F). Aplanospores of *Chlamydomonas* sp. were often surrounded by the small cells, most clearly observed after the cells were stained with DAPI (Fig. 1K).

In brown snow samples, a high abundance of *Chloromonas cryophila* (formerly *Scotiella cryophila/S. polyptera/S. nivalis* (H. –R. Preisig, pers. communication), and an enormous abundance of plant pollen, (approximately 50 to 100 micrometers in diameter; Fig. 1C), were observed. The types of eukaryotic cells, which were very abundant in red and brown snow, were rare in white snow samples, while scanning electron microscopy (SEM) showed various sizes of microbial cells (Fig. 1G, Fig. 1H, and Fig. 1I). Some of these microbial cells were in dividing stages (Fig. 1G and Fig. 1H). We observed a high abundance of fungal hyphae (Fig. 1J) in the snow samples. The greenish snowfield was dominated by large numbers of *Chloromonas nivalis* vegetative cells (Fig. 1L).

Colored snow was not present at the higher altitudes. The Jungfraujoch samples consisted only of white snow, ice core and the air isolates. The air isolates were dominated by yellow and pink pigmented colonies, whereas creamy, white-pigmented colonies appeared less frequently. After 2 days of incubation, there were 2 to 11 colonies per plate (from 140 N-liters of air absorbed).

3.2. DNA extraction, PCR amplification and RFLP analysis of the 16S-rDNA or 18S-rDNA inserts

We extracted the genomic DNA from the total community of the snow samples, ice and the air isolates, which yielded good templates for the PCR amplification. In order to obtain a representative overview of the community diversity, we used different primer sets targeting organisms of the eukaryotic, bacterial, and archaeal domains. In addition, we used the universal primers to amplify the ssu rRNA genes of both eukaryotic and prokaryotic organisms.

PCR amplification using different primer sets yielded expected ssu rRNA gene fragments. The PCR amplification with eukaryotic primers yielded a single band PCR product of approximately 1'750 bp from all snow samples. The universal primers yielded double-band amplicons, with estimated sizes of 1'000 bp and 880 bp, respectively, from the white, red and brown snow samples. The bigger size originated from the amplification of the 18S rRNA gene of eukaryotic organisms, whereas the smaller one was the amplification product of the 16S rRNA gene of the prokaryotic organisms. Archaeal primers yielded no PCR amplification products from all Jöri snow samples (several repeats), whereas the positive control and the JFJ snow samples yielded a clear single band. The bacterial primers amplified the bacterial 16S rRNA gene and yielded a single DNA band with an approximate size of 1'500 bp. PCR products obtained with the bacterial primer sets from red snow samples gave high band intensities, whereas there was less amplification product from brown and white snow samples.

PCR products obtained with eukaryotic, universal and archaeal primer sets from all samples were used for the construction of clone libraries. The PCR products obtained with bacterial primers from the red snow samples were also cloned into *E. coli* cells. A total of 11 different libraries were constructed, i.e. 7 libraries from Jöri snow samples and and 4 clone libraries from the JFJ samples, respectively. Detailed information of the cloning, number of clones obtained and analysed is summarized in Table 1.

Different cloning frequencies were obtained by using different primer sets. Cloning of the ssu rDNA inserts amplified by eukaryotic primers yielded lower cloning frequencies than inserts amplified by the universal or archaeal primers. Most of the colonies of the eukaryotic clone libraries appeared green on the selection medium indicating that they did not have inserts in their plasmid vector. This is probably caused by the failure in the ligation of PCR products (DNA inserts) and vector plasmids. More clones were obtained by using the archaeal, bacterial and universal primer sets than by using eukaryotic primers.

46 isolates and more than 400 positive clones that contained almost full or partial lengths of the ssu rRNA gene were analyzed. RFLP was done for screening and comparative analysis of the unique clones. The products from whole cell PCR were digested with 2 restriction endonucleases of *Hae*III and *Hinf*I. The snow clone libraries revealed more than 120 unique RFLP patterns. RFLP analysis allowed us to select a number of representative snow organisms covering the entire diversity observed. These were sequenced and subjected to a more rigorous phylogenetic analysis (Table 3 and 4).

3.3. Phylogenetic affiliation of the Jöri snow communities

Phylogenetic affiliation of the representative clones of the Jöri snow communities is depicted in Fig. 2a and Fig. 2b. Universal primer sets retrieved the 16S rRNA genes of the bacterial and 18S rRNA genes of the eukaryotic organisms. Therefore, sequences amplified with universal primers were affiliated to both, bacterial and eukaryotic domains. In contrast, the eukaryotic primer set specifically amplified eukaryotic 18S rDNAs and the bacterial primer set amplified only the bacterial 16S rDNAs (including mitochondria and chloroplast rDNA of eukaryotic organisms). Generally, the sequences of snow organisms that were affiliated to the domain bacteria were closely related to sequences retrieved from environmental clones of uncultured organisms rather than of cultivated ones.

The majority of the red and brown snow clones from Jöri corresponded to the *beta-Proteobacteria* subgroup. In this group, they formed several clusters within the families of Oxalobacteriaceae and Comamonadaceae. Sequence analysis of the representative snow clones showed similarity values between 94% and 99% to their closest neighbors presently existing in the databank (Table 3). They are closely related to either clones or isolates retrieved from cold environments, such as ARKICE and ARKMP clones from the Arctic, or Arctic isolates i.e. ARK10172, *Aquaspirillum arcticum*. One unique white snow clone affiliating to the family Comamonadaceae was closely related (99%

similarity) to an Antarctica microbial mat isolate strain R-7724 (AJ440986). Some clones in the *beta-Proteobacteria* affiliated to other environmental clones originating from soil, sediment or from aquifer environments.

Some Jöri snow clones were only distantly related to the presently existing sequences in the databank, suggesting that they belong to previously unknown phylogenetic groups. For example, a red snow clone RS 8-Bact59 was distantly related to clone UP9 from acidic forest soil with 93% sequence similarity and to the sequence of the *Herbaspirillum rubrisubalbicans* (AF137508) an Oxalobacteriaceae (*beta-Proteobacteria*) with a similarity value of 97%. Clone RS 8-Bact43 was distantly related (93%) to the descibed relative of *Collimonas fungivorans* (AJ496444). This bacterium belongs to the chitinolytic Oxalobacteriaceae and possesses an ability to grow on intact fungal hyphae (GenBank description). *Gammaproteobacteria* represented a minor part of the total clone libraries. One unique clone from the white snow sample, represented by clone WS 3-Uni06 was closely affiliated to the *Methylobacter* sp. strain SV96, AJ414655 within the family of Methylococcaceae (99% sequence similarity).

We obtained nonproteobacterial clones corresponding to the lineages of *Verrucomicrobia, Actinobacteria, Bacteroidetes, Cyanobacteria* (belonging to the same group as chloroplasts) and mitochondria. A phylotype that affiliated to the *Verrucomicrobia* was the white snow clone WS 3-Uni12. It was only distantly related (93% sequence similarity) to the clone CL120-133 (AF316730), a bacterioplankton from an ultra-oligotrophic crater lake. The brown snow clone BS 0-Uni07-B was distantly related (84% sequence similarity) to a Chrysophyceae *Chrysodidymus synuroideus* mitochondrion DNA.

The Jöri clones corresponding to the *Actinobacteria* and the *Bacteroidetes* were represented in the libraries of the red, white and brown snow samples. In the group of *Actinobacteria*, these snow clones made several clusters with representative isolates which have been found in glacier ice cores (Christner et al. 2003; Miteva et al. 2004) such as *Rhodococcus* sp., *Mycobacterium* sp., and *Propionibacterium granulosum*. Two other phylotypes that were closely related to an oligotrophic crater lake clone CL 500-95, were distantly related to the sequence of known-species. They were represented by the clone BS 0-Uni09 that was distantly related (91% sequence similarity) to the *Sporichtya polymorpha* strain IFO 12702, and clone WS 3-Uni10 that showed a low sequence similarity (90%) to the *Cellulomonas cellasea* strain DSM 20118.

In the *Bacteroidetes* group, our snow clones fall into different clusters. Clones RS 8-Uni11 and RS 8-Uni68-B were distantly related (91 to 92% similarity) to *Flexibacter canadensis* strain IFO 15130 (AB078046). Clones WS 3-Uni01, WS 3-Uni14 were closely related to *Flavobacterium ferrugineum* (M62798), whereas clone RS 8-Uni10 was distantly related to *Flexibacter filiformis* strain IFO 15056, AB078049 (91% similarity). The brown snow clones BS 0-Uni02, BS 0-Uni14-B and a red snow clone RS 8-Uni69-B were closely related to the pink-pigmented bacterium *Flectobacillus speluncae* strain GWF20B from biofilms of a spring cave, AY065626.

In the bacterial red snow libraries, many clones (38% or 22 out of 58 clones) were closely related (98 to 99% similarity) with the sequence of the black pine *Pinus thunbergii* chloroplast DNA. These sequences revealed various RFLP patterns and have different lengths, ranging from 1'440 to 1'500 bp. The other major fractions of the clones were 14 clones (24%) closely related to *Aquaspirillum arcticum*, strain IAM 14963, 9 clones (16%) were related to an Arctic isolate ARK10172, whereas 8 clones (14%) were related to a soil isolate EC4.

The Jöri snow sequences belonging to the eukaryotic domain were mostly affiliated to known-species as their closest relatives: Chlorophyta, Spermatophyta, Fungi, and Cercozoa (Dawson and Pace 2002). In the brown and red snow libraries using universal primers, we also obtained sequences that affiliated to the Chlorophyta.

Some representatives of snow algal sequences were obtained from our brown and red snow clone libraries. Clone BS 0-Uni11-B was closely related to the *Chloromonas* sp. 18S rDNA (AF514406) or *Chlamydomonas nivalis* (U57696) with the same sequence similarity of 98%. The next closest neighbor of this clone was *Chloromonas nivalis* (AF514409) with a lower similarity value (96%). A red snow clone RS 8-Uni41-B was distantly related to the *Chloromonas* sp. strain 047-99 (AF514406) with 93% similarity. The resting spores of these snow algae have red carotenoids to protect them from strong UV radiation on the snow surface.

Eukaryotic 18S rDNA clones affiliated to the Fungi were found in the white, brown and red snow. The closest neighbor of a brown snow clone BS 7-E11 was *Rhodotorula yarrowii* (AB032658). The closest relative of the red snow clone RS 8-Uni30 is *Zymoxenogloea eriophori* (U77063). A clone from the white snow library, represented by WS 10-E15, is distantly related (93% similarity) to *Spizellomyces acuminatus* (M59759).

Several Jöri clones revealed unusual base insertions in the middle of their rDNA sequences and exhibited a low sequence similarity to any known sequences. For example, the white snow clones of WS 3-Uni02 which is distantly related to the *Tetrahymena thermophila* mitochondrion (86% sequence similarity) and WS 3-Uni 05 that possess only 96% similarity to the *Monosiga ovata* 18S rRNA gene. Although these sequences might indicate unknown organisms, we did not include them in the phylogenetic trees prepared for this study.

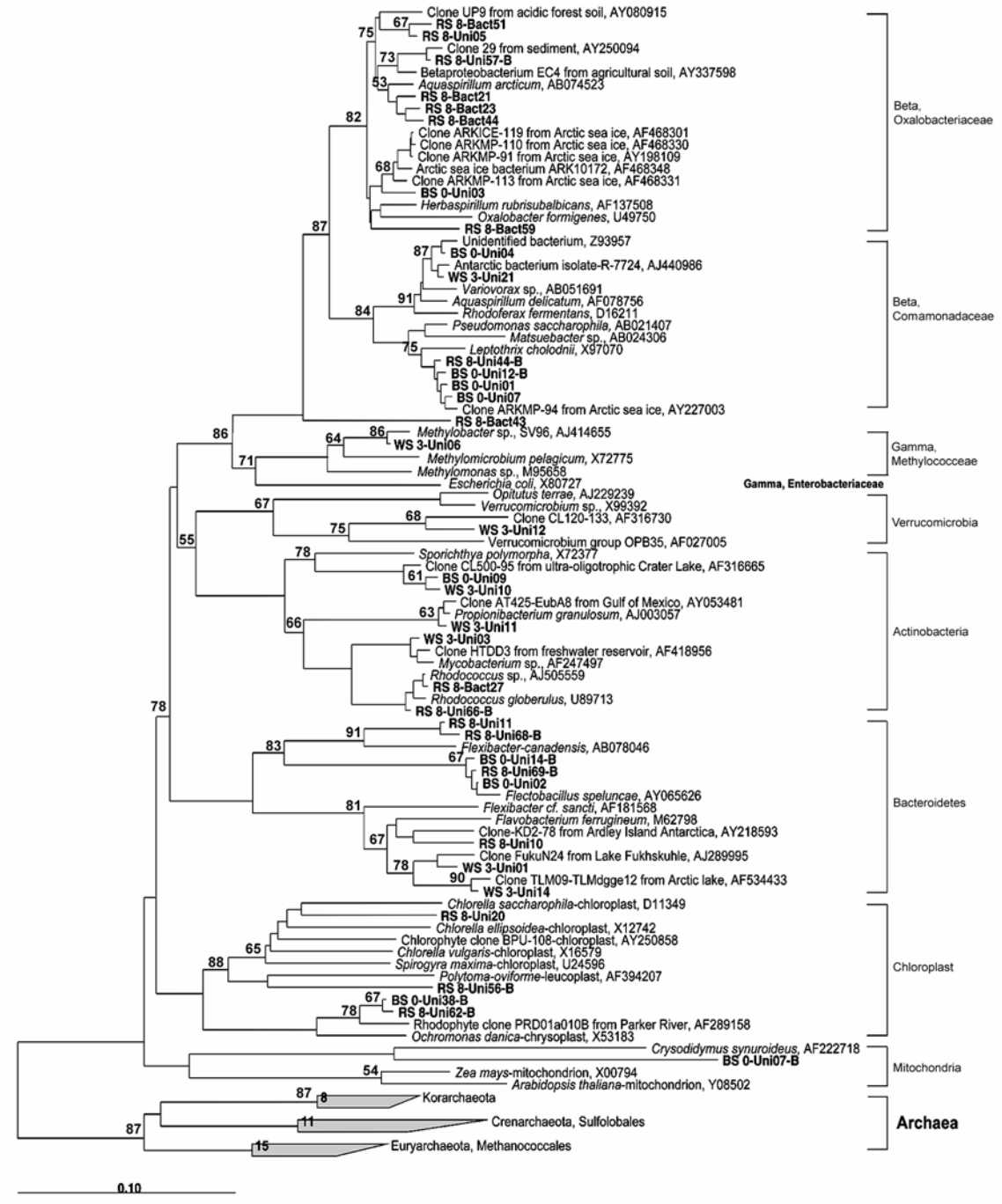

Figure 2a. Phylogenetic affiliation of the representative prokaryotic clones of the red snow (RS), brown snow (BS) and white snow (WS) communities from habitats near Lake Jöri XIII based on 16S rRNA gene comparisons. New sequences are shown in bold. The archaeal domain serves as an outgroup. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar indicates 10% sequence divergence.

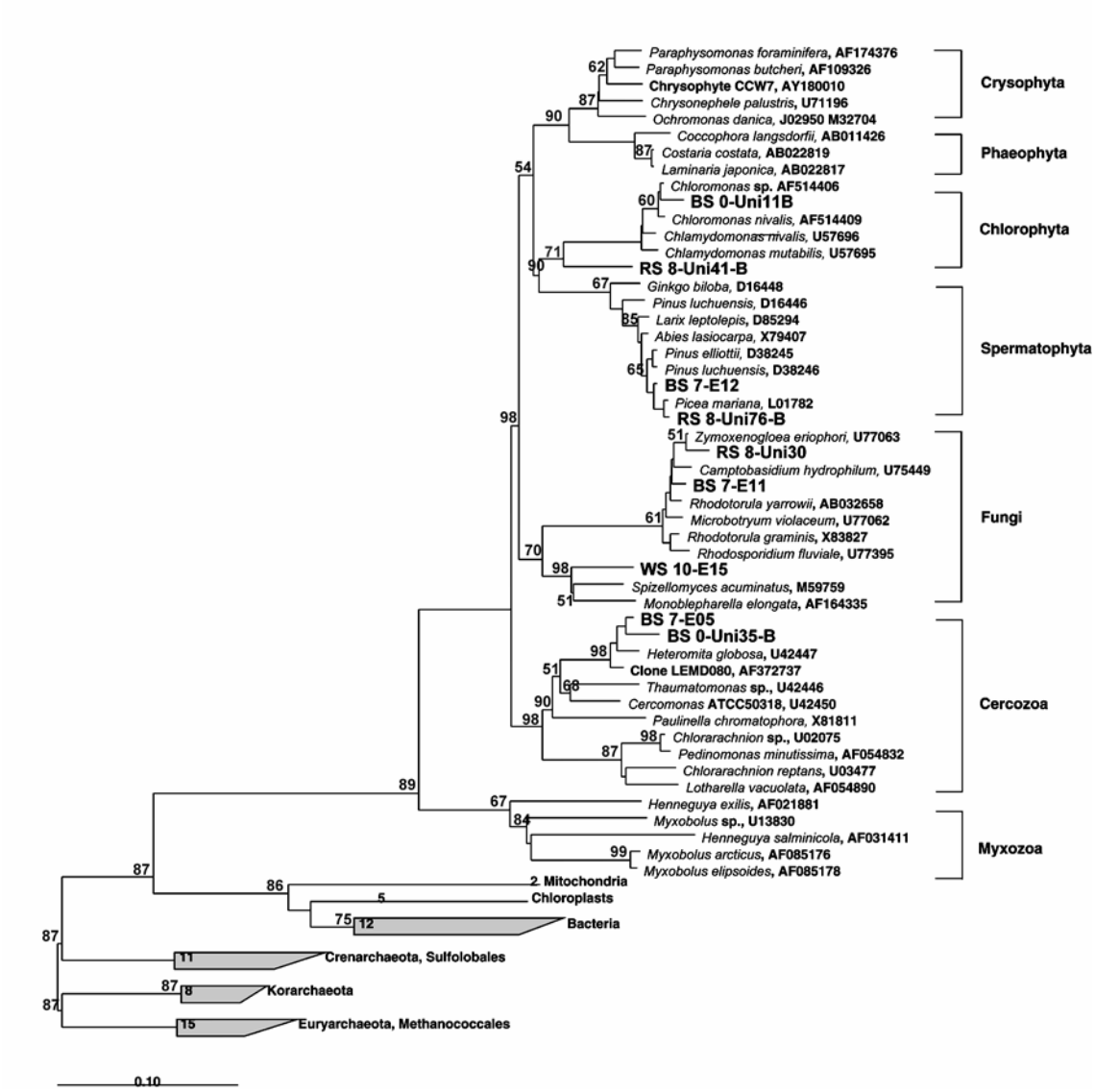

Figure 2b. Phylogenetic affiliation of the representative eukaryotic clones of the red snow (RS), brown snow (BS) and white snow (WS) communities from habitats near Lake Jöri XIII. New sequences are shown in bold. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The archaeal domain serves as an outgroup. The scale bar indicates 10% estimated sequence divergence.

Table 3. Identification of representative snow community clones from habitats nearby Lake Jöri XIII and their closest relatives obtained from BLAST search.

Clone (s) designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest relative described, accession no.	% Similarity
WS 3-Uni05	AJ867611	1059	Choanoflagellida; Codonosigidae	*Monosiga ovata* 18S ribosomal RNA gene, AF271999	Strain Russian	96		
WS 10-E02	AJ867629	1688	Fungi, Chytridiomycetes	*Spizellomyces acuminatus* 18S ribosomal RNA gene, M59759	Strain 62A	93		
WS 10-E14	AJ867630	1746	Fungi, Chytridiomycetes	*Spizellomyces acuminatus* 18S ribosomal RNA gene, M59759	Strain 62A	93		
WS 10-E15	AJ867631	1748	Fungi, Chytridiomycetes	*Spizellomyces acuminatus* 18S ribosomal RNA gene, M59759	Strain 62A	93		
BS 7-E04	AJ867632	1760	Cercozoan, Heteromites	Uncultured cercozoan clone LEMD080 ssu rRNA, AF372737	Eukaryotic from anoxic environment	96	*Heteromita globosa* 18S ribosomal RNA gene, U42447	96
BS 7-E05	AJ867633	1762	Cercozoan, Heteromites	Uncultured cercozoan clone LEMD080 ssu rRNA, AF372737	Eukaryotic from anoxic environment	97	*Heteromita globosa* 18S ribosomal RNA gene, U42447	97
BS 7-E06	AJ867634	1760	Cercozoan, Heteromites	Uncultured cercozoan clone LEMD080 ssu rRNA, AF372737	Eukaryotic from anoxic environment	96	*Heteromita globosa* 18S ribosomal RNA gene, U42447	96
BS 7-E11	AJ867635	1756	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	97		
BS 7-E12	AJ867636	1765	Viridiplantae; Pinaceae	*Abies lasiocarpa* 18S rRNA gene, X79407	Strain Hook	98		
RS 8-E35	AJ867637	1751	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	97		
RS 8-Uni03	AJ867638	1062	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	98		
RS 8-Uni21	AJ867639	1062	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	97		
RS 8-Uni22	AJ867640	1064	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	98		
RS 8-Uni26	AJ867641	1062	Fungi; Urediniomycetes	*Rhodotorula yarrowii* 18S rRNA gene, AB032658	Strain JCM 8232	97		
RS 8-Uni30	AJ867642	1045	Fungi; Urediniomycetes;	*Zymoxenogloea eriophori* ssu rRNA gene,U77063	Strain DJM 463-SS6	98		
BS 0-Uni11-B	AJ867643	1041	Chlorophyta	*Chloromonas* sp. 18S rRNA gene, AF514406	Strain 047-99	98	*Chloromonas nivalis*, AF514409 *Chlamydomonas nivalis,* U57696	96 98
BS 0-Uni35-B	AJ867644	1057	Cercozoan, Heteromites	Uncultured cercozoan LEMD080 ssu rRNA, AF372737	Eukaryotic from anoxic environment	95	*Heteromita globosa* 18S rRNA gene, U42447	95
RS 8-Uni41-B	AJ867645	1046	Chlorophyta	*Chloromonas* sp. 18S rRNA gene, AF514406	Strain 047-99	93		
RS 8-Uni52-B	AJ867646	1046	Fungi; Urediniomycetes	*Zymoxenogloea eriophori* ssu rRNA gene, U77063	Strain DJM 463-SS6	98		
RS 8-Uni76-B	AJ867647	1047	Viridiplantae, Pinaceae	*Abies lasiocarpa 18S rRNA gene,* X79407	Strain Hook	99		
BS 0-Uni01	AJ867678	866	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Leptothrix cholodnii* strain CCM 1827*,* X97070	97

Table 3. Continued

Clone (s) designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no	Source	% Similarity	Closest relative described, accession no.	% Similarity
BS 0-Uni02	AJ867679	868	Bacteroidetes; Sphingobacteriales	*Flectobacillus speluncae*, AY065626	Strain GWF20B from biofilm from a spring cave	99		
BS 0-Uni03	AJ867680	867	Betaproteobacteria, Oxalobacteraceae	Uncultured bacterium clone ARKMP-113, AF468331	Arctic sea ice	97	*Aquaspirillum arcticum* strain IAM 14963, AB074523	95
BS 0-Uni04	AJ867681	871	Betaproteobacteria; Comamonadaceae	Clone T3, Z93957	Activated sludge	99	*Variovorax* sp. HAB-30, AB051691	98
BS 0-Uni07	AJ867682	867	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila* strain 5-1, AF36875	97
BS 0-Uni09	AJ867683	879	Actinobacteria; Sporichthyaceae	Clone CL500-95, AF316665	Bacterioplankton from ultra-oligotrophic Crater Lake	99	*Sporichtya polymorpha* strain IFO 12702, X72377	91
BS 0-Uni10	AJ867684	879	Actinobacteria; Sporichthyaceae	Clone CL500-95, AF316665	Bacterioplankton from ultra-oligotrophic Crater Lake	99	*Sporichtya polymorpha* strain IFO 12702, X72377	91
BS 0-Uni12	AJ867685	867	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila* strain 5-1, AF36875	97
BS 0-Uni20	AJ867686	868	Bacteroidetes; Sphingobacteriales	*Flectobacillus speluncae*, AY065626	Strain GWF20B from biofilm from a spring cave	99		
WS 3-Uni01	AJ867696	875	Bacteroidetes, Flexibacteriaceae	Clone FukuN24, AJ289995	Lake Fukhskuhle	97	*Flavobacterium ferrugineum,* M62798	91
WS 3-Uni02	AJ867704	819	Alveolata; Ciliophora	*Tetrahymena thermophila* mitochondrion, AF396436	Strain SB210	86		
WS 3-Uni03	AJ867697	875	Actinobacteria; Mycobacteriaceae	Clone HTDD3, AF418956	Freshwater reservoir with metal-rich particles	98	*Mycobacterium rhodesiae* strain JS60, AF498650	98
WS 3-Uni06	AJ867698	873	Gammaproteobacteria, Methylococcaceae	*Methylobacter* sp., AJ414655	Strain SV96.	99		
WS 3-Uni10	AJ867699	867	Actinobacteria; Cellulomonadaceae	Clone CL500-95 , AF316665	Bacterioplankton from ultra-oligotrophic crater lake	99	*Cellulomonas cellasea* DSM 20118, X79459	90
WS 3-Uni11	AJ867700	872	Actinobacteria; Propionibacteriaceae	Clone AT425_EubA8, AY053481	Gulf of Mexico gas hydrates	99	*Propionibacterium granulosum* DSM 20700, AJ003057	98
WS 3-Uni12	AJ867701	882	Verrucomicrobia;	Clone CL120-133, AF316730	Bacterioplankton from ultra-oligotrophic crater lake	93	*Candidatus Xiphinematobacter rivesi*, AF217461	87
WS 3-Uni14	AJ867702	871	Bacteroidetes; Flexibacteraceae	CloneTLM09/TLMdgge12a, AF534433	Toolik Lake, Arctic	99	*Flavobacterium ferrugineum,* M62798	91
WS 3-Uni21	AJ867703	869	Unclassified bacteria	Isolate R-7724, AJ440986	Microbial mat, McMurdo Dry Valleys, Antarctica	99	*Aquaspirillum delicatum* strain LMG 4328, AF078756	98
RS 8-Uni05	AJ867705	865	Betaproteobacteria; Oxalobacteraceae.	Clone ARKMP91, AY198109	Arctic sea ice	98	*Aquaspirillum autotrophicum* strain IAM 14942, AB074524	97
RS 8-Uni10	AJ867706	871	Uncultured bacteria	Clone KD2-78, AY218593	From Ardley Island, Antarctica	94	*Flexibacter filiformis* strain IFO 15056, AB078049	91
RS 8-Uni11	AJ867707	870	Bacteroidetes; Sphingobacteriaceae	*Flexibacter canadensis*, AB078046	Strain IFO 15130	92		
RS 8-Uni12	AJ867708	863	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	98		

Table 3. Continued

Clone (s) designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest relative described, accession no.	% Similarity
RS 8-Uni20	AJ867717	899	Cyanobacteria, organelles	Clone BPU108, AY250858	Cryptoendolithic, McMurdo Dry Valleys, Antarctica	89	*Chlorella mirabilis* plastid DNA for 16S-like, X65100	87
RS 8-Uni23	AJ867709	868	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	98		
RS 8-Uni25	AJ867710	865	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	98		
RS 8-Bact02	AJ867648	1503	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	98		
RS 8-Bact17	AJ867656	1638	Uncultured bacteria	Clone RCP2-17, AF523878	Clone from a forested wetland	97	*Rickettsia* sp. strain HA-91, L36219	94
RS 8-Bact21	AJ867657	1506	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	98		
RS 8-Bact22	AJ867649	1416	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	99		
RS 8-Bact23	AJ867667	1495	Proteobacteria; Oxalobacteraceae	Isolate ARK10172, AF468348	Arctic sea ice	97	*Herbaspirillum lusitanum* strain P6-12, AF543312	95
RS 8-Bact24	AJ867650	1440	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	99		
RS 8-Bact27	AJ867668	1510	Actinobacteria; Nocardiaceae	*Rhodococcus sp*., AJ505559	Isolate 4115	99		
RS 8-Bact29	AJ867669	1491	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	97		
RS 8-Bact32	AJ867670	1493	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	97		
RS 8-Bact33	AJ867651	1476	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	99		
RS 8-Bact35	AJ867652	1441	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	99		
RS 8-Bact43	AJ867671	1489	Uncultured bacteria	Clone ARKICE-119, AF468301	Arctic sea ice	94	*Collimonas fungivorans*, str. CTE118, AJ496444 Beta, Oxalobacteraceae	93
RS 8-Bact44	AJ867672	1485	Betaproteobacteria, Oxalobacteraceae.	Isolate EC4, AY337598	Strain EC4 from agricultural soil	96	*Herbaspirillum lusitanum,* AF543312	95
RS 8-Bact51	AJ867673	1493	Betaproteobacteria, Oxalobacteraceae.	Clone ARKMP-110, AF468330	Arctic sea ice	96	*Herbaspirillum seropedicae* strain DSM 6445, ATCC 35892, Y10146	95
RS 8-Bact57	AJ867674	1492	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	97		
RS 8-Bact59	AJ867675	1469	Betaproteobacteria; Oxalobacteraceae.	Clone UP9, AY080915	Methylotroph populations in an acidic forest soil	93	*Herbaspirillum rubrisubalbicans* strain ICMP 5777, AF137508	97
RS 8-Bact61	AJ867676	1479	Betaproteobacteria; Oxalobacteraceae.	*Aquaspirillum arcticum,* AB074523	Strain IAM 14963	97		
RS 8-Bact62	AJ867653	1448	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	98		
RS 8-Bact63	AJ867654	1442	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	99		

Table 3. Continued

Clone (s) designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest relative described, accession no.	% Similarity
RS 8-Bact67	AJ867655	1453	Viridiplantae; Pinaceae	*Pinus thunbergii* chloroplast DNA, D17510	Chloroplast genome of the black pine *Pinus thunbergii*	98		
RS 8-Bact69	AJ867677	1490	Uncultured bacteria	Clone ARKMP-110, AF468330	Arctic sea ice	96	*Herbasprillum seropedicae* ATCC 35892, Y10146	94
BS 0-Uni03-B	AJ867687	883	Betaproteobacteria; Oxalobacteraceae	Clone ARKMP94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila,* AF368755	97
BS 0-Uni07-B	AJ867688	916	Organelle, Chrysophyceae	*Chrysodidymus synuroideus*, AF222718	*Chrysodidymus synuroideus* mitochondrion	84		
BS 0-Uni12-B	AJ867689	880	Betaproteobacteria; Oxalobacteraceae	Clone ARKMP94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila,* AF368755	97
BS 0-Uni14-B	AJ867690	883	Bacteroidetes	*Flectobacillus speluncae*, AY065626	Strain GWF20B	98		
BS 0-Uni27-B	AJ867691	882	Betaproteobacteria; Oxalobacteraceae	Clone ARKMP113, AF468331	Arctic sea ice	98	*Aquaspirillum arcticum,* AB074523	97
BS 0-Uni33-B	AJ867692	880	Bacteroidetes	*Flectobacillus speluncae*, AY065626	Strain GWF20B	98		
BS 0-Uni36-B	AJ867693	881	Betaproteobacteria; Oxalobacteraceae	Clone ARKMP94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila,* AF368755	97
BS 0-Uni37-B	AJ867694	883	Betaproteobacteria; Oxalobacteraceae	Clone ARKMP94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila,* AF368755	97
BS 0-Uni38-B	AJ867695	870	Rhodophyta	Uncultured rhodophyte PRD01a010B, AF289158	Parker River, Massachusetts	96	*Ochromonas danica* chrysoplast 16S rRNA, X53183	91
RS 8-Uni44-B	AJ867711	879	Betaproteobacteria; Oxalobacteraceae	Isolate ARK10157, AF468345	Arctic sea ice	97	*Aquaspirillum autotrophicum*, strain IAM 14942, AB074524	97
RS 8-Uni56-B	AJ867718	878	Chlorophyta	*Polytoma oviforme* 16S rRNA gene, AF374188	Strain SAG 62-27	86		
RS 8-Uni57-B	AJ867712	881	Betaproteobacteria, Oxalobacteraceae	Uncultured bacterium, AY250094	Napthalene-contaminated sediment	98	*Janthinobacterium agaricidamno-sum* strain SAFR-022, AY167838	98
RS 8-Uni62-B	AJ867719	868	Rhodophyta	Uncultured rhodophyte PRD01a010B, AF289158	Parker River, Massachusetts	96	*Ochromonas danica* chrysoplast 16S rRNA, X53183	90
RS 8-Uni66-B	AJ867713	888	Actinobacteria	*Rhodococcus erythropolis* 16S rRNA gene, AF532870	Strain W2	99		
RS 8-Uni68-B	AJ867714	884	Bacteroidetes	*Flexibacter canadensis*, AB078046	Strain:IFO 15130	91		
RS 8-Uni69-B	AJ867715	876	Bacteroidetes	*Flectobacillus speluncae*, AY065626	Strain GWF20B, from biofilm in spring cave	98		
RS 8-Uni72-B	AJ867720	870	Rhodophyta	Uncultured rhodophyte PRD01a010B, AF289158	Parker River, Massachusetts	96	*Ochromonas danica* chrysoplast 16S rRNA, X53183	91
RS 8-Uni75-B	AJ867716	886	Actinobacteria	*Rhodococcus erythropolis* 16S rRNA gene, AF532870	Strain W2	99		
RS 8-Uni80-B	AJ867721	870	Rhodophyta	Uncultured rhodophyte PRD01a010B, AF289158	Parker River, Massachusetts	96	*Ochromonas danica* chrysoplast 16S rRNA, X53183	90

3.4. Phylogenetic affiliation of the Jungfraujoch (JFJ) communities

Phylogenetic affiliation of the JFJ air isolates and snow communities is presented in Fig. 3a, whereas Fig. 3b shows the affiliation of the JFJ ice community. The RFLP patterns of the JFJ snow libraries showed lower diversity banding patterns compared to those of the Jöri clones. These results indicate that the snow communities at higher altitude habitats are comprised of phylogenetically less diverse microbial communities than those of the lower altitude "colored snow" habitats. The snow habitat at higher altitudes are also dominated by certain species, which are absent from the Jöri samples. The RFLP patterns of the bacterial clone library of JFJ ice showed lower diversity banding patterns (8 patterns out of a total of 66 clones analysed), whereas its universal clone library showed more diverse RFLP patterns (10 patterns out of a total of 38 clones analysed).

Table 4 lists the closest relatives and the distribution of the isolates and clone libraries from JFJ samples at different taxonomic lineages (bacteria, eukarya and archaea) based on their 16S and 18S rDNA identification. Almost all of the sequences of the JFJ snow communities were closely related to sequence from known organisms. Interestingly, but not surprising, some of JFJ air isolates and clone sequences corresponded to bacteria known as animal and human pathogens and commensals, which we did not obtain from the JFJ ice core sample. Isolate JFJ-Iso-11 was closely related (99%) to *Enterococcus faecium* (AY172570), whereas isolates represented by JFJ-Iso-29 were related (97%) to *Staphylococcus hominis* (L37601) isolated from the human blood culture. The snow clone JFJ-WS-Uni-02 was closely related to (98%) *Mesorhizobium* sp. (AY230775), a bacterium known as a human pathogen (GenBank descriptions). None of the sequences originating from the Jöri lakes catchment, which is relatively free from the anthrophogenic influences, had closest relatives to microorganisms of human origin. One unique sequence from JFJ snow corresponded to the snow alga *Chloromonas pichinchae*, eventhough a snow-coloring mass appearance event has never occurred at this high altitude.

All of the archaeal clones of JFJ snow communities affiliated to Crenarchaeota. One sequence JFJ-WS-Arch-03, from the archaeal snow library, was excluded because it revealed a possible chimera detected by the CHECK CHIMERA program. 39% of the total archaea libraries, represented by the clone JFJ-WS-Arch-07, was closely related (98%) to the clone HTA-E7 (AF418935) isolated from the freshwater reservoir. The rest of the archaeal clones corresponded by 97 to 99% similarities to the clone SCA1150 (U62812), which was isolated from soil. These clones were JFJ-WS-Arch-02, JFJ-WS-Arch-04, JFJ-WS-Arch-16 and JFJ-WS-Arch-18, which comprised 6%, 3%, 23% and 29% of the clone library, respectively

A high proportion of the isolates and snow clones of the JFJ communities corresponded to the *Actinobacteria*, whereas most sequences in the Jöri snow communities affiliated to the *beta-Proteobacteria* subgroup. A high proportion (88%) of the JFJ snow library were 99% similar to the sequence of *Rhodococcus erythropholis* (AF532870), a bacterium originating from the rhizosphere. In

addition, 37% of our isolates were 99% similar to the sequence of *Rhodococcus* sp. (AF181689), a cold-tolerant *Actinobacterium* isolated from Antarctica. Snow clones representing the *alpha-, beta-* and *gamma-Proteobacteria* i.e. clones JFJ-WS-Uni-02, JFJ-WS-Uni-06, JFJ-WS-Uni-41 and JFJ-WS-Uni-09 accounted only for 1 to 4 % of the total clone library. However, we obtained many yellow, orange or pink pigmented air isolates (33%) corresponding to the *alpha-Proteobacteria* of the genus *Sphingomonas* sp. which was also present in an Antarctic endolithic community. *Rhodococcus* and *Sphingomonas* genera were reported to be representatives of other permanently cold environments (Miteva et al. 2004).

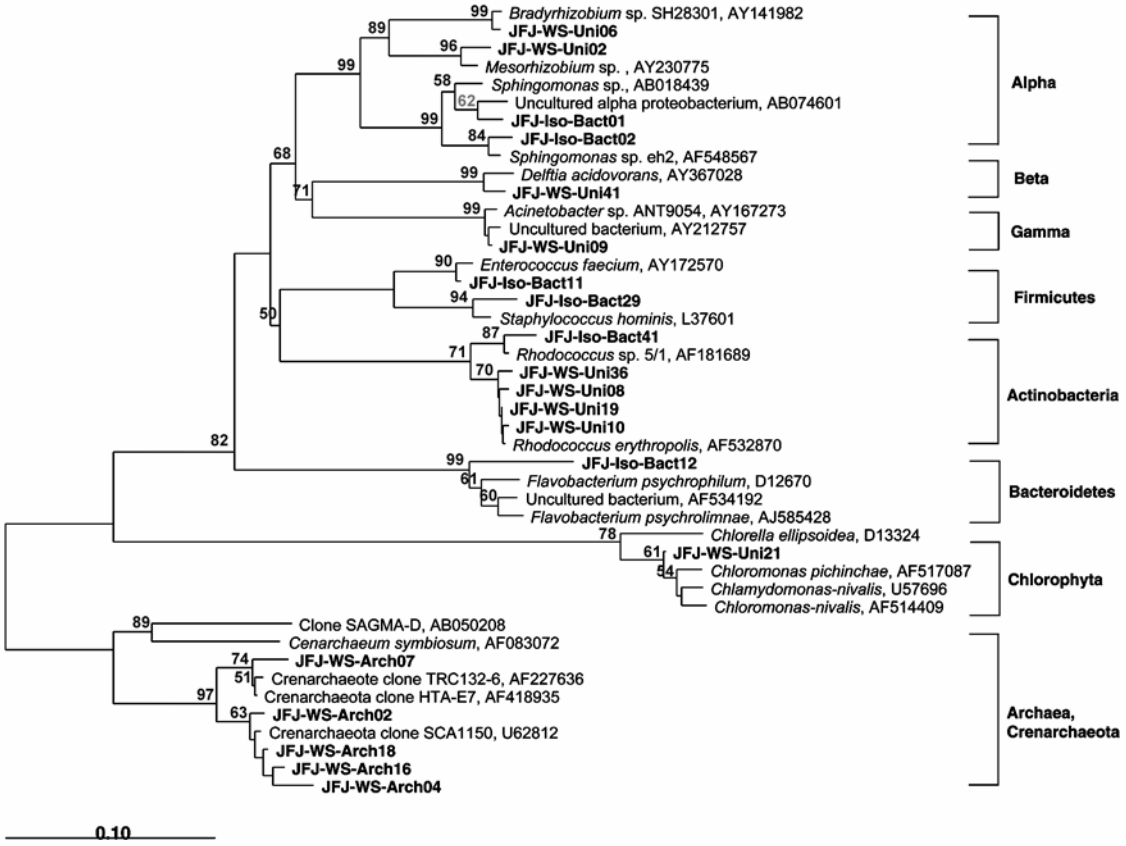

Figure 3a. Phylogenetic affiliation of the representative prokaryotic and eukaryotic clones of Jungfraujoch white snow community. Jungfraujoch snow clones are coded by **JFJ-WS-xxx,** whereas **JFJ-Iso-xxx** denotes an isolate. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar indicates 10% estimated sequence divergence.

All of the clones obtained with bacterial primers from JFJ ice affiliated to the *Proteobacteria* group, i.e *alpha-, beta-,* and *gamma-Proteobacteria* subgroups. In contrast to the snow community, the majority of clones of the JFJ ice community related to the Comamonadaceae of the *beta-Proteobacteria* subgroup (60 clones, accounted for 91% of the library), with sequence similarities between 94 to 99% to the sequences available in the databank. The closest known-species of the

clones within this family were *Aquaspirillum delicatum, Matsuebacter chitosanotabidus, Leptothrix* sp., and *Variovorax* sp. A small proportion of bacterial clones in the JFJ ice (6 clones, about 9%) belonged to the *alpha-* and *gamma-Proteobacteria* subgroups, within the family of Acetobacteraceae and the Xanthomonadaceae, respectively.

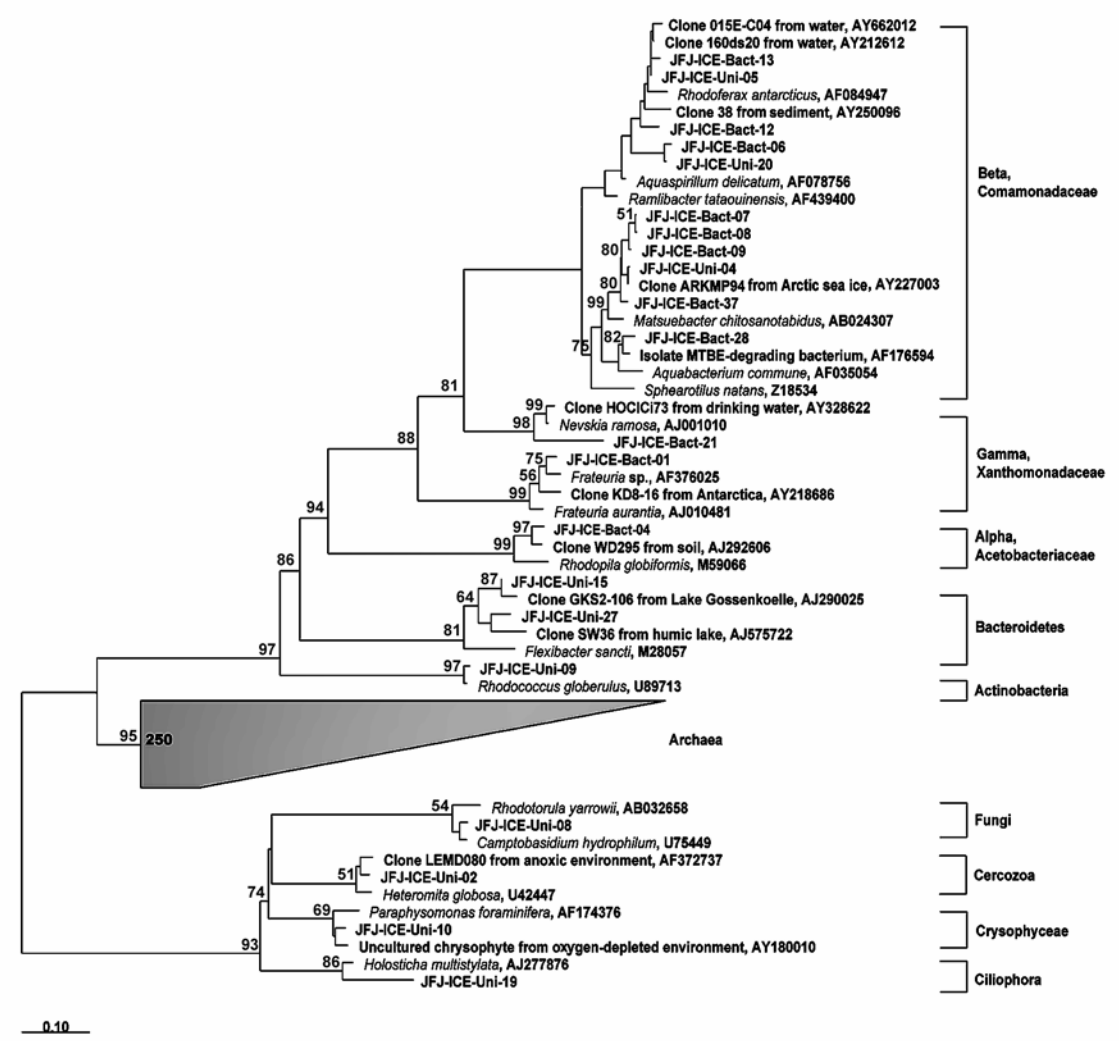

Figure 3b. Phylogenetic affiliation of the representative prokaryotic and eukaryotic clones of Jungfraujoch ice community. Jungfraujoch ice clones are coded by **JFJ-ICE-xxx**. Bootstrap values of 50% or higher from the neighbor-joining analysis are shown on the concerning branches. The scale bar indicates 10% estimated sequence divergence.

Compared to the clones obtained with bacterial primers, the phylogenetic distribution of the universal library from the JFJ ice community was broader. Three representative phylotypes belonging to the Comamonadaceae of the *beta-Proteobacteria* subgroup, which comprised 20 clones (53% of the library). Two other phylotypes were affiliated to the prokaryotic domain, i.e. to the *Bacteroidetes* group, whereas only 1 phylotype corresponded to the *Actinobacteria*. We obtained 4 clones in the JFJ ice community (11%) corresponded to the *Bacteroidetes* group, which were related to the clone

.

Table 4. Identification of air isolates, snow and ice clones from Jungfraujoch and their closest relatives obtained from BLAST search.

Isolate/Clone designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest described, accession no.	% Similarity
JFJ-Iso-Bact01	AJ867722	850	Alphaproteobacteria; Sphingomonadaceae	Clone APe4_19, AB074601	Environmental sample	97	*Sphingomonas* sp. strain S213	95
JFJ-Iso-Bact02	AJ867723	858	Alphaproteobacteria; Sphingomonadaceae	*Sphingomonas* sp. eh2, AF548567	Antarctic microbial endolithic community	98		
JFJ-Iso-Bact11	AJ867724	713	Firmicutes; Enterococcaceae	*Enterococcus faecium*, AY172570	Isolate related to pig/human fever outbreak in Jiangshu (China)	99		
JFJ-Iso-Bact12	AJ867725	871	Bacteroidetes; Flavobacteriaceae	Clone Glu1, AF534192	Soil	97	*Flavobacterium psychrolimnae* from Lake Fryxell mat, Antarctic	96
JFJ-Iso-Bact29	AJ867726	826	Firmicutes	*Staphylococcus hominis* ATCC 27844, L37601	Human blood culture	97		
JFJ-Iso-Bact41	AJ867727	852	Actinobacteria;	*Rhodococcus sp. 5/1*, AF181689	Cold tolerant alkane-degrading *Rhodoccocus* sp. from Antarctica	99		
JFJ-WS-Arch02	AJ867728	793	Unidentified Crenarchaeota	Clone SCA1150, U62812	Soil	97	None	
JFJ-WS-Arch03	AJ867729	760	Unidentified Crenarchaeota	Clone SCA1150, U62812	Soil	97	None	
JFJ-WS-Arch04	AJ867730	750	Unidentified Crenarchaeota	Clone SCA1150, U62812	Soil	98	None	
JFJ-WS-Arch07	AJ867731	791	Unidentified Archaea	Clone HTA-E7, AF418935	Metal-rich particles, freshwater reservoir	98	None	
JFJ-WS-Arch16	AJ867732	788	Unidentified Crenarchaeota	Clone SCA1150, U62812	Soil	98	None	
JFJ-WS-Arch18	AJ867733	790	Unidentified Crenarchaeota	Clone SCA1150, U62812	Soil	98	None	
JFJ-WS-Uni02	AJ867734	851	Alphaproteobacteria; Phyllobacteriaceae	*Mesorhizobium sp. Smarlab BioMol 2302657*, AY230775	Human pathogen	98		
JFJ-WS-Uni06	AJ867735	853	Alphaproteobacteria; Bradyrhizobiaceae	*Bradyrhizobium* sp., AY141982	Strain SH28301	99		
JFJ-WS-Uni08	AJ867736	854	Actinobacteria	*Rhodococcus erythropolis,* AF532870	Bacteria degrading N-AHL from rhizosphere	99		
JFJ-WS-Uni09	AJ867737	849	Gammaproteobacteria; Acinetobacter.	Clone wet54, AY212757	Equine manure	99	*Acinetobacter* sp. ANT9054, from Antarctic ice	99
JFJ-WS-Uni10	AJ867738	850	Actinobacteria	*Rhodococcus erythropolis,* AF532870	Bacteria degrading N-AHL from rhizosphere	99		
JFJ-WS-Uni19	AJ867739	834	Actinobacteria	*Rhodococcus erythropolis,* AF532870	Bacteria degrading N-AHL from rhizosphere	99		
JFJ-WS-Uni21	AJ867742	1039	Chlorophyta; Chlorophyceae	*Chloromonas pichinchae* 18S ssu rDNA, AF517087	Strain CU298B from snow	98		
JFJ-WS-Uni36	AJ867740	853	Actinobacteria	*Rhodococcus erythropolis,* AF532870	Bacteria degrading N-AHL from rhizosphere	99		
JFJ-WS-Uni41	AJ867741	846	Betaproteobacteria; Comamonadaceae	*Delftia acidovorans*, AY367028	Strain As3-4 from environmental	98		
JFJ-ICE-Bact-01	AJ867747	1537	Gammaproteobacteria; Xanthomonadaceae	Clone KD8-16, AY218686	Ardley Island, Antarctica	96	*Frateuria sp.acidophilic* Norwegian copper mine	97

Table 4. Continued

Isolate/Clone designation	Accession no.	Length (bp)	Phylum	Closest relative, accession no.	Source	% Similarity	Closest described, accession no.	% Similarity
JFJ-ICE-Bact-04	AJ867748	1481	Alphaproteobacteria, Acetobacteraceae	Clone WD295, AJ292606	Soil	97	*Acidisphaera sp.* strain NO-15	96
JFJ-ICE-Bact-06	AJ867749	1527	Betaproteobacteria, Comamonadaceae	Clone GKS16, AJ224987	Lake Gossenkoelle	98	*Polaromonas vacuolata*	97
JFJ-ICE-Bact-07	AJ867750	1507	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Matsuebacter chitosanotabidus*	96
JFJ-ICE-Bact-08	AJ867751	1506	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila*	96
JFJ-ICE-Bact-09	AJ867752	1484	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	98	*Matsuebacter chitosanotabidus*	95
JFJ-ICE-Bact-12	AJ867753	1509	Betaproteobacteria, Comamonadaceae	Clone D-16, AF523003	Natural mineral water	94	*Variovorax* sp. strain 6C-13	94
JFJ-ICE-Bact-13	AJ867754	1521	Betaproteobacteria, Comamonadaceae	Clone 015E-C04, AY662012	Groundwater contaminated of nitric acid-bearing uranium waste	97	*Aquaspirillum delicatum,* LMG 4328	96
JFJ-ICE-Bact-21	AJ867755	1516	Gammaproteobacteria, Xanthomonadaceae	Clone HOClCi73, AY328622	Drinking water distribution system simulator	92	*Nevskia ramosa*	92
JFJ-ICE-Bact-28	AJ867658	1523	Betaproteobacteria, Comamonadaceae	Isolate PM1, AF176594	Compost biofilter enrichments	97	*Leptothrix* sp.	97
JFJ-ICE-Bact-31	AJ867659	1516	Gammaproteobacteria, Xanthomonadaceae	Clone HOClCi73, AY328622	Drinking water distribution system simulator	92	*Nevskia ramosa*	92
JFJ-ICE-Bact-37	AJ867660	1523	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Matsuebacter chitosanotabidus*	96
JFJ-ICE-Uni-02	AJ867743	1057	Cercozoa (Protozoa)	Clone LEMD080, AF372737	Anoxic environments	97	*Spongomonas minima* strain ATCC 50404	96
JFJ-ICE-Uni-04	AJ867661	883	Betaproteobacteria, Comamonadaceae	Clone ARKMP-94, AY227003	Arctic sea ice	99	*Pseudomonas saccharophila*	97
JFJ-ICE-Uni-05	AJ867662	847	Betaproteobacteria, Comamonadaceae	Clone 160ds20, AY212612	Water	99	*Variovorax sp. 9726w*	99
JFJ-ICE-Uni-08	AJ867744	1040	Fungi, Urediniomycetes	*Rhodotorula yarrowii*, AB032658	Strain JCM 8232	97		
JFJ-ICE-Uni-09	AJ867663	852	Actinobacteria, Nocardiaceae	*Rhodococcus erythropolis*, X80618	Strain DSM43188T	100		
JFJ-ICE-Uni-10	AJ867745	1044	Stramenopiles, Chrysophyceae	Clone CCW7, AY180010	Uncultured chrysophyte from oxygen-depleted environment	97	*Synura uvella*	96
JFJ-ICE-Uni-15	AJ867664	841	Bacteroidetes, Flexibacteraceae	Clone GKS2-106, AJ290025	Lake Gossenkoelle	99	*Flexibacter* sp. strain CF1	91
JFJ-ICE-Uni-19	AJ867746	1051	Alveolata, Ciliophora	*Holosticha multistylata,* AJ277876	Genomic DNA	97		
JFJ-ICE-Uni-20	AJ867665	887	Betaproteobacteria, Comamonadaceae	Clone 38, AY250096	Napthalene-contaminated sediment	99	*Variovorax* sp. strain 35/28	99
JFJ-ICE-Uni-27	AJ867666	888	Bacteroidetes, Flexibacteraceae	Clone SW36, AJ575722	Lake Grosse Fuchskuhle	95	*Flexibacter filiformis* strain IFO 15056	91

GKS2-106 (AJ290025) from the Austrian Alpine Lake Gossenkölle and to the clone SW36 from the Lake Grosse Fuchskuhle (AJ575722). In contrast to the dominance of the *Actinobacteria* in the JFJ snow community, the affiliation of the ice clones within this group was only 3% of the library. In addition, we did not obtain any clone corresponding to organisms of animal or human-origin in the JFJ ice libraries. Ice clones representing the eukaryotic domain accounted for 16% (6 clones). These clones affilated to the *Fungi*, the *Cercozoa*, the *Stramenophile* (Crysophyceae family) and the *Alveolata* (Ciliophora).

4. Discussion

We used different types of snow and ice habitats at high altitudes as model ecosystems where extreme and highly fluctuating environmental conditions might select for particular microbial communities. In these ecosystems, the snow cover becomes a major habitat during the melting season. Previous studies provided information about abundance, biomass, composition, and size structure of the microbial communities thriving in high mountain habitats (Felip et al. 1995; Alfreider et al. 1996; Pernthaler et al. 1998; Glöckner et al. 1999). The microbial community diversity was not studied in great detail so far. To our knowledge, few studies were carried out addressing the composition and phylogenetic affiliation of microorganisms present in freshly deposited snow, in the melting snowpack and in newly formed ice (for example Felip et al. 1995; Wille et al. 1999).

The snow samples which we analyzed in this study contained a high abundance of mineral particles, as observed in the light microscope, as well as by scanning electron microscopy (SEM). These particles might play important roles in providing the essential nutrients to the temporary snow habitats. That microbes metabolized nutrients in the snow cover and in slush layers was demonstrated by the high uptake rates of labeled thymidine and leucine, which were higher than those were measured in lake water (Felip et al. 1995). The authors suggested that organic matter present in slush layers of the snow cover can originate from both, autochthonous and allochthonous sources, with autochthonous organics originating from the lake water and from degrading organisms. Since our samples were taken from the surface of the snow layer, allochthonous inputs and primary productivity are probably the more important contributors of nutrients. Allochthonous materials (mineral particles, vegetation debris, microorganisms) originating from deposition of airborne desert and soil dust are commonly observed on snow covers in the Alps (Psenner 1999).

Results from the sequenced clones demonstrated that the Jöri red snow contained communities which are more diverse compared to those in brown and white snow. Red snow communities affiliated into 17 different lineages, whereas the brown and white snow communities contained 10 different lineages. Many clones (38%) from Jöri snow were closely related to the family Pinaceae i.e *Pinus thunbergii, Abies lasiocarpa* and *Picea mariana*, species which were planted in an avalanche

protection forest at Stillberg, at about 10 km distance from Jöri. With bacterial primers, we obtained clones closely related to chloroplast 16S rDNA of the Pinaceae, whereas with eukaryotic and universal primers, we obtained clones of their 18S rDNA. In contrast, no sequences related to the Pinaceae were found in the JFJ snow libraries. These observations support the hypothesis that snow communities in mountain habitats far above the timberline are enriched by the deposition of particulates from far away. The uniqueness of the snow community is also accentuated by observations of Felip et al. (1995) who reported that the flagellate and cilliate communities in the high mountain snow cover differed from those in the communities of nearby lakes.

Most snow algal species found in the slush layer of the lake snow cover corresponded to the organisms present in the lake, whereas the *Chlamydomonas* sp. occured in a high density only in snow cover habitats (Felip et al. 1995). Some prokaryotic phylotypes found in snow communities belong to the most abundant bacterioplankton present in lake Jöri XIII at the end of the ice cover period. Six phylotypes retrieved from brown snow (i.e BS 0-Uni01, BS 0-Uni07, BS 0-Uni03-B, BS 0-Uni12-B, BS 0-Uni36-B, and BS 0-Uni37-B) corresponded to this clone and were closely related (99% sequence similarity) to the clone ARKMP-94 (AY227003) isolated from Arctic sea ice. In addition, a white snow clone WS 3-Uni21 showed close relatedness (99% similarity) to the isolate R-7724 from a microbial mat in McMurdo Dry Valley, Antarctica and to *Aquaspirillum delicatum* strain LMG 4328 (98% similarity). This phylotype was one of the most abundant bacterioplankton in early winter and interestingly it also corresponded to two biofilm clones from this lake (Yuhana, unpublished). These results suggest that the high mountain lake habitats are colonized by non-indigenous organisms, which appear in the snow cover. The well-adapted organisms might be able to thrive and dominate the communities under the harsh conditions.

The majority of the clones from snow and ice affiliated to the bacterial domain, in which the subgroup of the *beta-Proteobacteria* comprised the dominant fraction. These results are consistent with our previous investigations, i.e. a study in the pure culture collection (Chapter 2) and a study of the most abundant of the bacterioplankton in the lake (Chapter 3). Many other studies carried out in various aquatic habitats also revealed a global dominance of the *beta-Proteobacteria* in freshwater ecosystems (Hiorns et al. 1997; Crump et al. 1999; Glöckner et al. 1999; Glöckner et al. 2000; Brümmer et al. 2003).

An interesting feature of this study on the microbial diversity of high altitude cold ecosystems is that the largest proportions of the snow and ice clones had remarkable similarities with sequences reported from cold environments far away such as from the polar regions. 62% and 24% of JFJ ice clones obtained with bacterial and universal primers, respectively, were affiliated to the clone ARKMP-94 from Arctic sea ice (AY227003) with 98 to 99% sequence similarity. In addition, 48 % and 23% of the Jöri brown and red snow clones respectively, also corresponded to this clone. The largest fraction (33%) of the Jöri white snow libraries, corresponded to clone TLM09/TLMdgge12a (AF534433), retrieved from the Arctic Toolik Lake (99% similarity). Many other sequences affiliated

to clones or isolates from Antarctica and from Alpine lakes. Similar observations were made about the seasonal and spatial ditribution of the most abundant bacterioplankton from the lake water column (Chapter 3). These results support a hypothesis that the occurrence of related phylotypes of organisms from geographically diverse cold environments is possibly due to the similar strategies for survival and the ability to remain active at freezing temperatures (Priscu et al. 1998).

Interestingly, we also obtained clones from snow and ice which correspond to those retrieved from anoxic environments. A number of clones obtained from community DNA of Jöri brown snow and JFJ ice, amplified by eukaryotic or universal primers (BS 7-E04, BS 7-E05, BS 7-E06 and BS 0-Uni35-B) and JFJ-ICE-Uni-02 were closely related (96 to 97% similarity) to the uncultured cercozoan clone LEMD080 ssu rDNA. This eukaryotic clone was previously isolated from the anoxic environment by Dawson and Pace (2002) and had 96 to 97% sequence similarity to the isolate *Heteromita globosa* 18S rRNA gene (U42447). The authors consider the Cercozoa together with the Alveolata (31%), Fungi (26%), and Stramenopiles (18%), as ecologically important groups with anaerobic members. Cercozoa accounted for 10% of the total number (out of 125 clones) of eukaryotic phylotypes retrieved from 3 different anoxic environments.

Despite a high abundance of *Chlamydomonas nivalis* aplanospores observed by microscopy, its abundance in the brown and red snow clone libraries was only 3% and 1%, respectively. The proportion in the clone libraries not always represent the actual abundance in the environment, as can also be seen from DAPI stained samples (Fig. 1E and 1F). Prokaryotes are much more abundant in the community. We'd like to emphasize that frequencies by which clones appear in libraries should be interpreted carefully. Inefficiency of the initial cell lysis during DNA extraction may lead already to uneven DNA extraction and recovery from environmental samples (Rochelle et al. 1992; Picard et al. 1992; More et al. 1994; Rochelle et al. 1994). Variable numbers of rRNA genes per cell and of rRNA operons (Farrelly et al. 1995; Klappenbach et al. 2000; http://rrndb.cme.msu.edu) may influence template concentration in PCR, which can lead to the clone abundance in the libraries (Chandler et al. 1997). In addition, different PCR conditions can result in selective 16S rDNA amplification (Reysenbach et al. 1992; Suzuki and Giovannoni 1996) which also strongly influences the final proportion of clones in libraries.

Results of this study showed that permanently cold environments such as JFJ snow and ice habitats are colonizable for various organisms. However, we observed different microbial diversities in snow communities from different altitudes. Phylotype diversities expressed by the Shannon–Weiner (H', Hughes et al. 2001) diversity index to reveal phylotype richness showed value of H'=0.51 for the universal clone libraries of JFJ snow. Higher H' values are shown from the ice samples, i.e H'=2.02 for the JFJ ice community obtained with universal primers and H'= 1.18 is obtained with bacterial primers. The Shannon–Weiner diversity indices of the red, white and brown snow from the Jöri lakes catchment obtained with the universal primers were 2.47; 2.02, and 1.58, respectively. These indices indicate that generally the Jöri snow communities were more diverse than the JFJ communities.

Several environmental conditions may contribute to the differences such as the availability of liquid water, nutrients, UV radiation, and primary productivity. The enormous abundance of phototrophic snow algae present in the lower altitude habitats may contribute to the availability of nutrients and carbon sources available to a multitude of heterotrophs.

In conclusion, this study provides evidence for a high diversity and a broad phylogenetic affiliation of the snow and ice communities. The results are consistent with previous studies (Felip et al. 1995) that the snow and ice communities comprise complex trophic level relationships, in which the algae act as primary producer and bacteria support nutrient cycling (Sherr et al. 1992).

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Community shifts in response to variable environmental conditions

Abstract:

We studied microbial population composition shifts by exposing natural samples to variable simulated conditions. Samples of the water column were taken from Lake Jöri XIII (± 2'640 m a.s.l), an oligo- to mesotrophic cold water lake. Temporal temperature gradient gel electrophoresis (TTGE) of PCR-amplified 16S-rDNA was used to follow the community composition changes. The microbial populations responded to the variable conditions, as revealed by TTGE pattern shifts during the experiment.

1. Introduction

Remote, high mountain lakes are ecosystems with extremely variable fluctuations and often sudden changes. Microorganisms living in these habitats are challenged by harsh temperatures, between below freezing and +15ºC maximally, by seasonal and diurnal radiation differences between darkness and high levels of UV, by nutrient deprivation and bursts during snow melt phases, and by anoxia and darkness in the hypolimnion below a long lasting ice cover. During the short summer period, the epilimnion experiences high diurnal temperature fluctuations, strong light penetration and high UV radiation (Bothwell et al. 1994).

We have been interested in finding out how microbial population composition shifts in habitats which get exposed to variable physicochemical and trophic conditions. The bacterioplankton community composition and its changes were studied *in vitro* by simulating 3 environmental determinants. Culture-independent approaches and Temporal Temperature Gradient Gel Electrophoresis (TTGE) of PCR-generated DNA fragments were used in this study to follow the community composition changes. Small sub-unit 16S rRNA genes were used as comparative molecular markers (Ward et al. 1990). TTGE with PCR amplified DNA fragments allow us to detect complex structures of communities and their changes and to identify the microorganisms, including the non-culturable ones (Muyzer et al. 1993).

The three environmental determinants chosen i.e nutrient supply, radiation, and redox conditions strongly affect biological processes. Combinations of these 3 essential determinants were used in forced simulations. The results illustrate the population flexibility and further support the validity of the selective adaptation hypothesis for ecosystems under harsh and highly variable environmental conditions.

2. Materials and methods

2.1. Sample collection and experimental design

Samples were collected at 5 depths from Lake Jöri XIII (2'640 m a.s.l.; 10.4 m maximum depth) situated in the Alps of the Canton Graubünden, Switzerland. Sampling was carried out in October 2002. During this period, the lake was homothermic i.e. the lake water masses were completely mixed. Sampling was done with a 5 l Niskin sampler and the water samples were placed in sterile bottles and kept at low temperatures in a cooling box for transportation. Subsamples for the experiments under the different conditions were made under laboratory conditions (Table 1).

Table 1. Experimental overview of the community changes *in vitro*.

Sample [a] from depth (m)	Nutrients added [b]				No Nutrients			
	dark		light		dark		light	
	oxic	anoxic	oxic	anoxic	oxic	anoxic	oxic	anoxic
1	*		*		*		*	
3		*		*		*		*
5	*		*		*		*	
7		*		*		*		*
9	*		*		*		*	

*: experiments carried out
a) Samples were collected on 1st October, 2002
b) 10-fold diluted Luria Bertani medium

Volumes of 100 ml for each experiment were placed in 200 ml glass bottles. Samples were incubated at 4ºC under a combination of the following conditions: with or without additional nutrients, in the light and in the dark, with oxygen (stirring) and anoxically. The additional final nutrient concentrations used were 10 fold diluted Luria Bertani (LB) medium. A sample set was incubated in the dark (the bottles were wrapped in aluminum foil). Samples that needed light were incubated under two 15-Watt-tube lamps (Osram) in 30 x 30 x 70-cm^3 refrigerator, with a continuous light regime. Aerobic incubation was done on stirrer plates. Each bottle of this treatment was stirred continuously using a magnetic stirrer bar, the bottles were covered by sterile cotton plugs. For anoxic treatment, the bottles were purged with O_2-free N_2 gas (completely filled and tightly sealed). Resazurin was used as redox indicator in the anaerobic culture.

2.2. Nucleic acid extraction, PCR, and TTGE analysis for community profiling

After 2 weeks of incubation the cultures were used for the total genomic DNA extraction, PCR amplification, and TTGE analysis. The procedures were described in detailed previously (Chapter 3). Cells were collected by filtration using 0.22 μm pore-size type GVWP Millipore filters (Millipore, Volketswil, Switzerland). The sample aliquots varied, depending of the turbidity of the culture. 40 to 50 ml aliquots from the treatments with nutrients and 75 ml from cultures without added nutrients were filtered. The microorganisms on the filters were rinsed by the lysis buffer and further processed according to the methods recommended by the manufacturer (Qiagen Corp., Stanford, USA). The cells were lysed enzymatically by using lysozyme and disrupted physically by beat beating. Beat beating was done by adding 100 mg of glass beads (diameter 0.25 mm) to each tube and shaking at 80% of maximum speed in a model MM2000 bead-beater (Retsch, Haan, Germany) for 1 min. Samples were further treated according to the protocol recommended by the manufacturer (Qiagen Corp., Stanford, USA). Tubes containing extracted genomic DNA were stored at -20°C until further processing.

To amplify the 16S rRNA gene fragments from the total community DNA, we used the general bacterial primers S-D-Bact-0008-b-S-20 (5‘-AGA GTT TGA TCM TGG CTC AG) and S-D-Bact-1524-a-A-18 (5‘-AAG GAG GTG ATC CAR CCG) (Lane, 1991). PCR reactions were prepared in a volume of 25 μl, containing (final concentration) dH_2O, *Taq* buffer (1x) (Sigma), 0.1 mg ml^{-1} Bovine Serum Albumin DNase-free (Amersham Pharmacia Biotech Inc.), 0.2 mM dNTPs, 200 nM of each primer, 40 U ml^{-1} *Taq* Polymerase (Sigma), and approximately 50-100 ng template DNA. PCR was performed with a Techne Thermocycler (Techne LTD, Duxford Cambridge, U.K.). A set of touch down PCR running conditions was used as described previously (Chapter 3). PCR products were analyzed by electrophoresis in a 1% agarose gel and 0.5X TAE running buffer [45 mM Tris-acetate (pH 8.3), and 4 mM EDTA].

The primary PCR products were used as templates for the nested PCR. This method was done to increase the sensitivity and signal strength of the PCR amplification, which is a necessary step for community fingerprint analysis. Universal primers S-D-Bact-341-b-S-17 (5‘-CCT ACG GGA GGC AGC AG) and GC-Univ-907-a-A-20 (5‘-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCG TCA ATT CMT TTR AGT TT) (Lane, 1991; Muyzer et al., 1998) were used to amplify the 16S rRNA gene fragments (approximately 560 bp). PCR was performed by initial denaturation at 94°C for 5 min, followed by 75°C for 15 sec. The next steps were 20 cycles of 94°C for 20 sec, annealing at 65°C -lowered by 0.5°C every cycle- for 30 sec, and elongation temperature 72°C for 1 min. These steps were followed by 15 cycles of 94°C for 20 sec, 52°C for 30 sec, 72°C for 70 sec, and a final extension step at 72°C for 10 min.

TGGE was carried out in a DCode Mutation Detection system (Bio-Rad Laboratories). 10 μl of the PCR samples and 10 μl of the 2X loading buffer (70% [v/v] glycerol, 0.05% [w/v] bromophenol

blue) were loaded onto 6% polyacrylamide gels (acrylamide: N,N'-methylene bis-acrylamide 37.5:1 [w/w]; 7 M urea, 1X TAE). The gels were run at temperatures ranging from 54°C to 64°C, temperature ramping rate was 1.1°C h^{-1}, the voltage was 90 V (4.0 V/cm), and running time was about 9 h. The gels were stained in 1 μg ml^{-1} ethidium bromide solution for 15 min, destained in water for 45 min, visualized, and photographed under UV transillumination.

3. Results and Discussion

Community composition changes of the lake bacterioplankton incubated under variable simulated conditions are summarized in Fig. 1. In previous studies we were able to show that TTGE banding patterns of the bacterioplankton community sampled while the lake water column was turning over were essentially identical over the whole depths (Chapter 3). In this study TTGE banding patterns of the initial community from different depths revealed identical patterns. Initial TTGE profile from depth of 1 m is the same as those from depths of 3 m and 5 m, as well as from the depths of 7 m and 9 m. These community patterns from this shallow lake differ from those observed in meromictic lakes, in which the water column is constantly stratified (Casamayor et al. 2002; Bosshard et al. 2000; Hollibaugh et al. 2001).

TTGE analysis allows us to follow the changes in community composition (Muyzer et al. 1993; Muyzer and Smalla 1998; Muyzer 1999). The bands revealed in the TTGE gels indicated the most abundant microorganisms that can be amplified by PCR (Ogiers et al. 2002). In the present study, we illustrate that the community composition shifts in response to variable environmental conditions. Since the simulation treatments included 3 different determinants, one expects to observe different community patterns. After 2 weeks of incubation, the various community patterns shifted differently from their initial patterns (Fig. 1).

Based on the TTGE analysis presented in Fig. 1, it can be concluded that nutrient concentration is a strong determinant for community shifts. The community patterns did not get altered much from the initial patterns in cultures with low nutrient concentrations but the pattern is distinguishable from the one at the start of the experiment. In low nutrient medium, the presence or absence of oxygen and light did not yield very different banding patterns as compared to their initial patterns (designated as NDO, NLO, NDA and NLA at all depths, except 5 m). A few bands appeared or disappeared or got less intense under the different conditions. The only exception was the treatment NDO from 5 m depth, which shifted differently and which is more similar to HDO rather than to NLO. Under low nutrient conditions, the majority of TTGE bands still appeared, some were got less intense after the 2 week incubation period. A few bands are missing at the end of the experiment (i.e. the first two bands which migrated shortest in the gel).

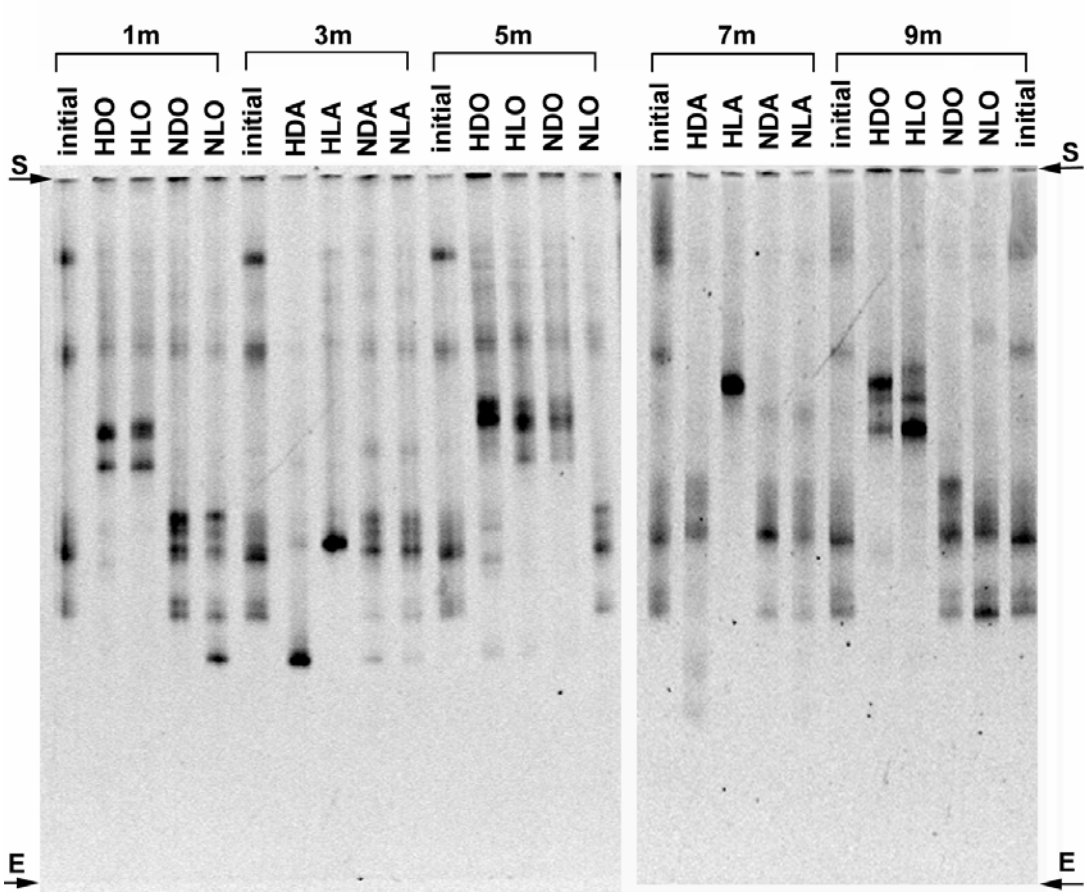

Figure 1. TGGE patterns of total bacterial community DNA of Lake Jöri XIII from different depths treated under variable environmental conditions and incubated *in vitro*.

Initial: initial population before treatments were applied. H: 10-fold diluted LB medium added, N: no nutrients added, D: incubation in the dark, L: incubation in the light, A: anoxic condition, O: oxic condition. S: start of gels, E: end of gels.

A low microbial diversity develops when the community is exposed to high nutrient concentrations and anoxic conditions. This can be seen from experiments designated HDA and HLA at depths of 3 m and 7 m, respectively. In these treatments light is an essential determinant. The community exposed to the HDA treatment may be dominated by anaerobic heterotrophs, whereas in the HLA treatment anoxigenic phototrophic organisms might begin to develop. Generally, it can be seen that incubations under high nutrient conditions showed fewer bands than the initial community and also fewer when compared to those incubated at low nutrient concentrations. Low numbers of TTGE bands indicate a limited number of species, i.e. only those microorganisms which possess the highest competitive ability, are selected from the community. Environments with low nutrients lead to

a more diverse species composition, but the community shifts between the initial and the final state were less pronounced and cultures kept in the light did not yield distinctly different patterns.

Community shifts carried out with planktic communities which were sampled at different seasons from the Lake Jöri XIII habitat showed pronounced dynamic changes of the bacterioplankton compositions (Chapter 3). The communities were composed of a large number of unique species, which are phylogenetically diverse and which respond to various environmental fluctuations.

The results presented in this study suggest that community competition and adaptation processes are common in ecosystems which get exposed to environmental fluctuations. Physiological flexibility of the microorganisms present in the „community store“ allows the community to rapidly respond to environmental changes and thus to ensure the functioning of microbially-mediated processes in the habitat. Microorganisms possessing a high physiological flexibility, so-called niche generalists, will dominate in their communities under various conditions (Elena and Sanjuán 2003), while niche specialists will get selected under more specific conditions. Environmental changes express themselves as physical and chemical instabilities, which exert selection pressures for different microorganisms. These changes promote the dynamic microbial community transitions in fluctuating environments (Rainey and Travisano 1998).

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I taught and was a teaching assistant in the "Microbial Diversity Course-Bio III" (2 weeks in 2001 to 2005) and in the "Microbial Ecology Course" (5 weeks, 2000 to 2004).

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- Plant Molecular Biology (June-July 2001, 5 weeks) with Beat Keller.
- Molecular Phylogeny (June-July 2002, 5 weeks) with Elena Conti.
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I presented my work orally at the International Agouron Geobiology Course (2002), Plant Biology Workshops (2001 and 2004) and at the Meeting of the Swiss Society for Microbiology in Lugano (2004); and with posters at the International Symposium "Water in the Alps" in Davos (2002), at the Annual Meeting of Swiss Society for Microbiology in Basel (2003) and in Lugano (2004).